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# Materials Science & Engineering C

## Biobased dynamic hydrogels by reversible imine bonding for controlled release of thymopentin --Manuscript Draft--

<b>Manuscript Number:</b>	MSEC-D-21-01195R1
<b>Article Type:</b>	Research Paper
<b>Keywords:</b>	O-carboxymethyl chitosan; Imine bonding; Dynamic hydrogel; Thymopentin; drug release; Density functional theory
<b>Corresponding Author:</b>	Suming Li University of Montpellier Montpellier, France
<b>First Author:</b>	Suming Li
<b>Order of Authors:</b>	Suming Li Rui Yu Eddy Petit Mihail Barboiu Wenjing Sun Congmei Chen
<b>Abstract:</b>	<p>Thymopentin (TP5) is widely used in the treatment of autoimmune diseases, but the short in vivo half-life of TP5 strongly restricts its clinical applications. A series of blank and TP5 loaded hydrogels were synthesized via reversible dual imine bonding by mixing water soluble O-carboxymethyl chitosan (CMCS) with a dynamer (Dy) prepared from Jeffamine and benzene-1,3,5-tricarbaldehyde. TP5 release from hydrogels was studied at 37°C under in vitro conditions. The molar mass of CMCS, drug loading conditions and drug content were varied to elucidate their effects on hydrogel properties and drug release behaviors. Density functional theory was applied to theoretically confirm the chemical connections between TP5 or CMCS with Dy. All hydrogels exhibited interpenetrating porous architecture with average pore size from 59 to 83 μm, and pH-sensitive swelling up to 10 000 % at pH 8. TP5 encapsulation affected the rheological properties of hydrogels as TP5 was partially attached to the network via imine bonding. Higher TP5 loading led to higher release rates. Faster release was observed at pH 5.5 than at pH 7.4 due to lower stability of imine bonds in acidic media. Fitting of release data using Higuchi model showed that initial TP5 release was essentially diffusion controlled. All these findings proved that the dynamic hydrogels are promising carriers for controlled delivery of hydrophilic drugs, and shed new light on the design of drug release systems by both physical mixing and reversible covalent bonding.</p>
<b>Suggested Reviewers:</b>	thierry Delair thierry.delair@univ-lyon1.fr  elmira arab-tehrany elmira.arab-tehrany@univ-lorraine.fr  katarzyna jelonek kjelonek@cmpw-pan.edu.pl
<b>Response to Reviewers:</b>	Dear editor and reviewers, Many thanks to the reviewers for their pertinent comments. We revised our manuscript accordingly and highlighted in red the changes in the revision. A point-to-point reply is given. We hope the paper is now acceptable for publication. Thanks. Sincerely yours, Suming LI

## **Re: Manuscript Number: MSEC-D-21-01195**

Dear editor,

Many thanks to the reviewers for their pertinent comments. We revised our manuscript accordingly and highlighted in red the changes in the revision. A point-to-point reply is given below.

We hope the paper is now acceptable for publication. Thanks.

Sincerely yours,

Suming LI

### **Responses to the comments of Referee 1**

This manuscript reported the preparation of the dynamic hydrogel by reversible imine bond crosslinking for sustained release of thymopentin. The mechanism is also confirmed by density functional theory. Therefore, I recommend its publication, but some revisions should be addressed before publication:

**Comment 1:** “The Abstract is not attractive and tedious, which looks like the conclusion. Please revise it.”

**Response:** Thanks for this comment. The whole abstract is revised accordingly and marked in red.

**Comment 2:** “The mechanical properties of hydrogels should be supplied such as tensile strength and modulus et al. In addition, the optical photograph should also be presented.”

**Response:** We agree with the reviewer that mechanical properties such as the tensile strength and modulus are important parameters of hydrogels. In the case of our dynamic hydrogels, they are “soft” hydrogels and used as carriers for in vitro drug release. As shown in Fig. 3, the storage modulus ( $G'$ ) varies from hundreds to 2300 Pa, and is stable in the studied strain/frequency range. Moreover, TP5-loaded hydrogels maintained their structural integrity after drug release for 7 days. Therefore, rheological analysis can elucidate the feasibility of these dynamic hydrogels in drug release. Currently, we're focusing on obtaining better malleability of hydrogels for applications as wound dressing, and related tensile strength and modulus measurements will be conducted.

The as prepared hydrogels are transparent, and the optical photograph has been shown in a previous paper [1].

**Comment 3:** “Is the drug uniformly distributed in the hydrogel? How could you prove that?”

**Response:** Thymopentin is a highly hydrophilic drug [2, 3], and its saturation concentration in water is above 100 mg/mL. For all cases of TP5-loaded hydrogels in this manuscript (0.1 mg/ml, 3 mg/ml, 9 mg/ml), the TP5 concentration is much lower than its saturation concentration. Moreover, TP5-loaded hydrogels were synthesized by in-situ encapsulation method. In other words, TP5 was homogeneously dissolved in the mixed aqueous solution of CMCS-Dynamer, and then gelation proceeded at 37 °C. Therefore, it can be assumed that TP5 is uniformly

distributed in the hydrogel volume.

**Comment 4:** “Please supply the drug loading capacity of hydrogels.”

**Response:** As mentioned in the Section 2.5 of the manuscript, in situ incorporation of TP5 results in complete drug encapsulation. Therefore, the drug loading capacity of TP5-loaded hydrogels is 100 %. Moreover, before in vitro release, all the TP5-loaded hydrogels were washed 3 times using PBS (pH 7.4), and the washing PBS solution was collected and analysed by HPLC-UV. No TP5 was detected, confirming that the TP5 was completely embedded in the hydrogel volume.

According to the reviewer’s comment, the following phrase is added at the end of Section 2.5 (Page 7) : “In situ incorporation of TP5 results in complete drug encapsulation. Therefore, the drug loading capacity of all the TP5-loaded hydrogels is 100 %.”.

## **References**

[1] R. Yu, Y. Zhang, M. Barboiu, M. Maumus, D. Noël, C. Jorgensen, S. Li, Carbohydr. Polym., 244 (2020) 116471.

[2] Y. Yin, D. Chen, M. Qiao, Z. Lu, H. Hu, J. Controlled Release, 116 (2006) 337-345.

[3] Y. Xu, G. Li, W. Zhuang, H. Yu, Y. Hu, Y. Wang, Journal of Materials Chemistry B, 6 (2018) 7495-7502.

## **Responses to the comments of Referee 2**

Authors prepared interesting biobased reversible imine hydrogels based on carboxymethyl chitosan, Jeffamine and benzene-1,3,5-tricarbaldehyde. The produced hydrogels are interesting but it is not clear how the presented system differs from the earlier work of the authors presented in ref 31-33. Is it only the application to TP5 loading and release? Authors should more clearly present the advancement to state of the art and novelty of the current work in relation to the previous works. Otherwise the work is relatively well-presented and organized and could be revised according to following points.

### **Response:**

Thanks for the reviewer’s pertinent comment. Similar hydrogels have been reported in our previous papers. This work focuses on the design of novel drug delivery systems by both physical mixing and reversible covalent bonding. Moreover, the effect of the molar mass of CMCS on the hydrogel properties and drug release behaviors is studied.

According to the reviewer’s comment, the following phrase is added: “CMCS with different molar masses of CMCS was used since the molar mass affects the physico-chemical properties of hydrogels.”.

**Comment 1:** “Introduction could be improved to better provide the background and important

state of the art and finally the hypothesis and novelty definition for the current work. Now the different paragraph feels separate and the "story" is not clear and fluent."

**Response:** Revision is made to better present the background and the state of the art (Pages 3, 4, 5). Two new references were cited (ref. 15, ref. 35).

The hypothesis and novelty of the manuscript were added in the last paragraph, Introduction part, according to the reviewer's comment.

**Comment 2:** "Page 5, 2.2: How was the concentration of remaining aldehyde groups measured?"

**Response:** As shown in note b) of Table 1: The concentration of Dy solution was obtained from the remaining aldehyde groups. In a typical reaction, 0.5 mmol BTA (81 mg) reacted with 0.5 mmol Jeffamine ED2003 (950 mg) to form a Dy solution. As BTA had 3 aldehydes and Jeffamine 2 amines, there remained theoretically 0.5 mmol of aldehydes in the dried Dy. Addition of 10 mL water yielded a dynamer solution of 0.05 M.

**Comment 3:** "Fig 1: The FT-IR evidence of TP5 incorporation in the hydrogels is very weak. The incorporation should be shown and quantified also by other means."

**Response:** We agree with the reviewer that the characteristic band at  $1512\text{ cm}^{-1}$  is rather weak for Gel80K-TP5. Nevertheless, this band is significant. As shown in Fig.1 a-b), Gel80K-TP5, Dy-TP5, and TP5 present this characteristic band at  $1512\text{ cm}^{-1}$  which is assigned to the N-H pending of amines in TP5. This band was not observed for the other samples, including Gel80K, Dy, and CMCS. This confirms the incorporation of TP5 in the hydrogel. Noticeably, the N-H band is weak because it is adjacent to the intense carbonyl groups band at  $1595\text{ cm}^{-1}$ . Moreover, as shown in Fig. 2, NMR was another efficient means to verify the imine bonding between TP5 and the Dynamer, thus indirectly evidence the attachment of TP5 in the hydrogel network. Compared with the spectrum of Dy, no signals in the range of 10.1-10.2 ppm were observed on the spectrum of Dy-TP5, implying that all the remaining aldehydes of Dy had reacted with the amine groups of TP5, in agreement with FTIR data (Fig. 1b).

**Comment 4:** "The chemical structure of the hydrogels should be better confirmed and characterized. Now only one small FT-IR spectrum is presented for Gel80K"

**Response:** As mentioned in Section 2.2, 2.4 and 2.5 in the manuscript, the hydrogel frameworks were crosslinked via dual imine bonding from CMCS, Jeffamine and BTA with well known structures. Therefore, FT-IR analysis is an efficient means for the characterization of samples by comparison of characteristic bands such as aldehyde, amine, and imine bands, as reported in our previous papers (ref. 33, 34 in manuscript).

**Comment 5:** "Fig 2. the NMR spectra. The chemical structures of the compounds or important chemical bonds should be drawn and the most important signals assigned in the NMR spectra."

**Response:** The chemical structures of all the compounds are shown in Scheme 1. Thus, they are not drawn in Fig. 2 as there is no space. Fig. 2 is largely modified. And in particular, assignment of all the signals of TP5 is given. Changes are made in Page 14 and Page 15.

**Comment 6:** "It would be interesting to add the pore diameter and especially to porosity of the

hydrogels.”

**Response:** According to the reviewer’s comment, the pore size distributions of as-prepared blank hydrogels are added in Fig. 4 and related discussion is added in Page 18 and Page 19.

**Comment 7:** “The mass loss ratios of the hydrogels is huge. How crosslinked are they, it seems crosslinking degree is very small? The original gel content of the hydrogels should be determined by extraction with suitable solvent.”

**Response:** It’s true that the mass loss ratio is high, especially in acidic medium due to the lower stability of imine bonds at low pH. In media around physiological pH, lower mass loss ratios are obtained. In the case of Gel80K, the mass loss ratio is only 30 % at pH 7.

As shown in Scheme 1, hydrogels are crosslinked by dual imine bonding in two steps: first between the aldehydes of BTA and the amines of Jeffamine (4 h at 70°C in methanol) to yield a dyanmer, and then between the remaining aldehydes of BTA and the amines of CMCS (24 h at 37°C in water). As shown in Table 1, the concentrations of CMCS and Dy in hydrogels are 1.8 and 4.3 w/v%, respectively. It’s probable that part of Dy is not involved in the hydrogel network, and is washed away during swelling, thus leading to relatively high mass loss as reported in our previous paper (ref. 33 in manuscript).

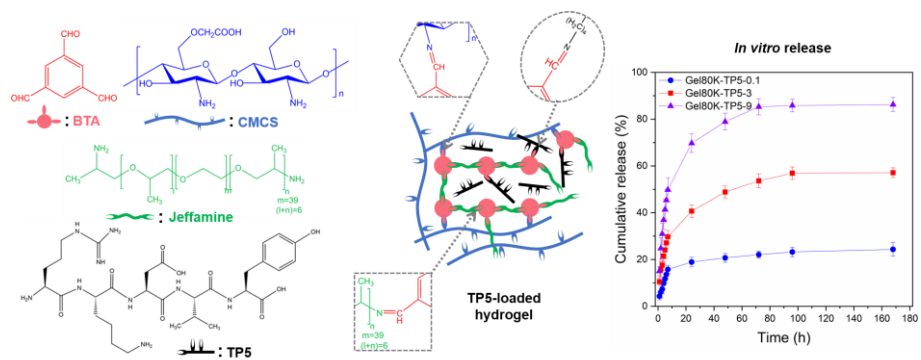
Concerning the determination of the original gel content of the hydrogels, the suitable solvent is water. This the original gel content can be considered as the remaining gel content after swelling 24 h at pH 7.4 because of the rapid swelling and mass loss (as shown in Fig. S4). According to the mass loss ratio after 24 h (Fig. 5b), the original gel contents are 64.5 % for Gel80K, 44.3% for Gel30K, and 46.5 % for Gel25K.

**Comment 8:** “The drug release rate is quite fast originally and also large amount of drug is not finally released. Is this really suitable for real applications?”

**Response:** We agree with the reviewer that in the first stage, the release rate is relatively fast. However, as described in the manuscript, TP5 was in-situ loaded in hydrogels via chemical and physical linkages, and TP5 release can be tuned by varying many parameters (e.g., drug load content, drug loading condition, pH of release medium, etc.). Although the burst release is fast, hydrogels can keep sustainable release up to 7 days. Thus, TP5 release would be further improved in the following work.

High unreleased amount of TP5 was obtained only for low loading concentration (0.1 and 3 mg/mL). When increasing the loading concentration to 9 mg/mL, the unreleased ratio decreases to 10 %. Therefore, by adjusting the loading condition, the unreleased rate can be tuned. On the other hand, TP5 release rate in pH 5.5 is much faster than that in pH 7.4 due to the fact that the imine bond is prone to hydrolyse in acidic condition (Fig. 7b). This would be very interesting for tumour therapy because of the slightly acidic pH in tumour. Therefore, these dynamic hydrogels crosslinked by dual imine bonding could be promising in cancer therapy by local administration.

- *O*-carboxymethyl chitosan-based dynamic hydrogels are synthesized by dual-imine bonding
- Thymopentin-loaded dynamic hydrogels are fabricated by *in-situ* encapsulation
- As-prepared hydrogels exhibit strongly pH-sensitive swelling behaviors
- *In vitro* release of thymopentin can be tuned by varying parameters, including pH of the release medium, drug load, and hydrogel preparation
- Density functional theory theoretically confirms the chemical connections between thymopentin and hydrogel network



Thymopentin was loaded in O-carboxymethyl chitosan-based hydrogels via physical mixing and imine bonding. *In vitro* release was investigated, and analyzed using Higuchi model and density functional theory computation.



### **Declaration of interest statement**

On behalf of all co-authors, I wish to confirm that there are no conflicts of interest associated with this publication untitled “Biobased dynamic hydrogels by reversible imine bonding for controlled release of thymopentin”, and there has been no significant financial support for this work that could have influenced its outcome.

Sincerely,

Suming LI

### Author statement

Rui Yu did the experiments and wrote the manuscript. Eddy Petit did the HPLC analyses. Mihail Barboiu participated in the design of the research. Suming Li conceived and supervised the research work, and revised the manuscript. Wenjing Sun conceived the Density functional theory (DFT) computation and wrote this part. Congmei Chen performed the the DFT computation.



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1 **Biobased dynamic hydrogels by reversible imine bonding for**  
2 **controlled release of thymopentin**

3

4 Rui Yu,<sup>1</sup> Eddy Petit,<sup>1</sup> Mihail Barboiu,<sup>1\*</sup> Suming Li,<sup>1\*</sup> Wenjing Sun,<sup>2\*</sup> Congmei Chen<sup>3</sup>

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15

1 **Abstract**

2 Thymopentin (TP5) is widely used in the treatment of autoimmune diseases, but the  
3 short *in vivo* half-life of TP5 strongly restricts its clinical applications. A series of blank  
4 and TP5 loaded hydrogels were synthesized via reversible dual imine bonding by  
5 mixing water soluble *O*-carboxymethyl chitosan (CMCS) with a dynamer (Dy)  
6 prepared from Jeffamine and benzene-1,3,5-tricarbaldehyde. TP5 release from  
7 hydrogels was studied at 37°C under *in vitro* conditions. The molar mass of CMCS,  
8 drug loading conditions and drug content were varied to elucidate their effects on  
9 hydrogel properties and drug release behaviors. Density functional theory was applied  
10 to theoretically confirm the chemical connections between TP5 or CMCS with Dy. All  
11 hydrogels exhibited interpenetrating porous architecture with average pore size from  
12 59 to 83 μm, and pH-sensitive swelling up to 10 000 % at pH 8. TP5 encapsulation  
13 affected the rheological properties of hydrogels as TP5 was partially attached to the  
14 network via imine bonding. Higher TP5 loading led to higher release rates. Faster  
15 release was observed at pH 5.5 than at pH 7.4 due to lower stability of imine bonds in  
16 acidic media. Fitting of release data using Higuchi model showed that initial TP5  
17 release was essentially diffusion controlled. All these findings proved that the dynamic  
18 hydrogels are promising carriers for controlled delivery of hydrophilic drugs, and shed  
19 new light on the design of drug release systems by both physical mixing and reversible  
20 covalent bonding.

21

22 Keywords: *O*-carboxymethyl chitosan; Imine bonding; Dynamic hydrogel;  
23 Thymopentin; Drug release; Density functional theory

24

## 1 **1. Introduction**

2 Thymopentin (TP5) is a synthetic pentapeptide (Arg-Lys-Asp-Val-Tyr,  $M_n = 679.77$ )  
3 associated with the residual sequence (32-36) of thymic hormone thymopoietin [1, 2].  
4 It possesses the biological properties characteristic of thymopoietin [3], and has been  
5 widely used for immunomodulation [4], and for treatments of autoimmune diseases [5],  
6 including cancer immunodeficiency [6], rheumatoid arthritis [7], tuberculosis [8],  
7 chronic heart failure [9], and chronic lymphocytic leukemia [10]. TP5 can facilitate the  
8 differentiation of thymocytes and affect the function to induce maturation of T-cells [11,  
9 12] and bone marrow dendritic cells [13]. Nevertheless, due to the short half-life ( $\leq 30$   
10 s in plasma) and extensive metabolism in the gastrointestinal tract [14], TP5 is generally  
11 applied as a freeze-dried powder by intramuscular or percutaneous injection [5].  
12 Chronic autoimmune diseases require long therapies and repetitive injections of TP5  
13 which could provoke complications. These disadvantages highly limit the clinical  
14 utilization of TP5.

15 It has been reported that the in vivo efficiency of TP5 can be improved by slow infusion  
16 [15]. Therefore, controlled drug delivery systems are considered as an appealing  
17 strategy to achieve slow infusion of TP5, including nanoparticles [16], micelles [17],  
18 liposomes [18], and hydrogels [19, 20]. These various systems present many  
19 advantages such as prolonged drug release, constant drug concentration, reduced side  
20 effects, protection of drugs from degradation, etc. Micelles, liposomes and  
21 nanoparticles have been used to encapsulate TP5, but the encapsulation efficiency is  
22 quite low because of its high hydrophilicity [17, 21]. Dissolving microneedle array was  
23 studied as a new strategy for TP5 delivery by transdermal injection [4]. However,  
24 customized microneedle arrays and daily administration are needed, leading to high  
25 cost and low bioavailability. In contrast, hydrogel delivery systems appear most  
26 interesting for sustained delivery of hydrophilic drugs, and have been used clinically  
27 [22]. Hydrogels can easily encapsulate a variety of hydrophilic therapeutic agents,  
28 including small-molecule drugs [23], macromolecular drugs [24] and cells [25].  
29 Furthermore, the high-water up taking capacity endows hydrogels with the physical  
30 features similar to tissues, and excellent biocompatibility. Besides, the crosslinked  
31 framework of hydrogels can hinder penetration of proteins [26], and thus protecting  
32 vulnerable bioactive drugs from degradation by inwardly diffusing enzymes [27].

33 Hydrogels have been investigated for the encapsulation of TP5. Zhang et al. developed

1 a physical hydrogel from polylactide-poly(ethylene glycol) (PLA-PEG) block  
2 copolymers via stereo-complexation between poly(L-lactide) and poly(D-lactide)  
3 blocks [19]. High drug loading and rapid drug release were obtained. Nevertheless,  
4 PLA degradation leads to acidic by-products which could provoke inflammatory  
5 reactions, and such physical hydrogels exhibit low stability and mechanical strength.  
6 Su et al. synthesized a hydrogel by crosslinking PLA grafted *O*-carboxymethyl chitosan  
7 (CMCS) using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and *N*-  
8 hydroxysuccinimide (NHS) as crosslinking agents [28]. The resulting hydrogel was  
9 used to encapsulate TP5 by immersion of dried gel in a TP5 solution. More than 80%  
10 of TP5 was released in 48 h. However, the presence of residual chemicals or solvents  
11 could compromise the biocompatibility of the system [29]. Therefore, it becomes a  
12 major challenge to develop a delivery system of TP5 which exhibits good  
13 biocompatibility, stability and high encapsulation efficiency.

14 CMCS is a water-soluble derivative of chitosan, the second most abundant  
15 polysaccharide in nature. It has been studied as a promising carrier for the delivery of  
16 peptides and proteins [30]. On the other hand, Jeffamine is a biocompatible  
17 polyetheramine containing poly(propylene oxide) (PPO) and poly(ethylene oxide)  
18 (PEO) blocks, as well as primary amino groups at chain ends. It has been used in drug  
19 delivery because of its water solubility and cytocompatibility [31]. Recently, our group  
20 has developed a CMCS and Jeffamine based dynamic hydrogel that could be potentially  
21 used for drug delivery [32], cartilage engineering [33], and antibacterial materials [34].  
22 The hydrogel was prepared via dual imine bond crosslinking between the amino groups  
23 of CMCS and the aldehyde ones of a 3D dynamer (Dy). Dy was first obtained by Schiff-  
24 base reaction of 1,3,5-benzenetri-aldehyde (BTA) and di-amino Jeffamine, followed by  
25 Schiff-base reaction of Dy and CMCS in aqueous solution to yield a 3D network.  
26 Trifunctional BTA served as a core to ensure 3D crosslinking, and Jeffamine as a linker  
27 to endow Dy with aqueous solubility. The gelation process is conducted in aqueous  
28 solution under mild conditions, which is beneficial for encapsulation of fragile drugs

1 such as proteins or peptides [22]. Different from other chitosan-based hydrogel by  
2 imine bonding [35], CMCS and Jeffamine based dynamic hydrogels exhibit regular and  
3 interconnected porosity which is beneficial for swelling, diffusion of physiological  
4 fluids, and drug encapsulation [33,34]. Interestingly, the hydrogel network contains free  
5 aldehyde and amino groups which are able to react with the functional groups of drugs  
6 or other bioactive molecules.

7 This work aimed to develop a novel delivery system of TP5 from CMCS and Jeffamine  
8 based dynamic hydrogels by in situ encapsulation. CMCS with different molar masses  
9 of CMCS was used since the molar mass affects the physico-chemical properties of  
10 hydrogels. *In situ* incorporation of TP5 within the dynamic hydrogel should allow to  
11 achieve complete drug encapsulation by physical adhesion to pore walls and reversible  
12 imine bonding to the hydrogel network via Schiff base reaction between the amino  
13 groups of TP5 and aldehydes of the dynamer. The rheological properties, internal  
14 morphology and swelling performance of dynamic hydrogels and TP5-loaded  
15 hydrogels were comparatively investigated. *In vitro* drug release of TP5-loaded  
16 hydrogels was studied under different conditions, and fitted using Higuchi's model.  
17 Finally, density functional theory (DFT) was applied to model the different imine bonds  
18 in the hydrogel network.

19

## 20 **2. Experimental section**

### 21 2.1 Materials

22 *O,O'*-bis(2-aminopropyl) poly(propylene glycol)-block-poly(ethylene glycol)-block-  
23 poly(propylene glycol), namely Jeffamine<sup>®</sup> ED-2003 with  $M_n$  of 1900, methanol (96%),  
24 hydrogen peroxide solution (H<sub>2</sub>O<sub>2</sub>, 30%) citric acid (99.5%), disodium hydrogen  
25 phosphate dodecahydrate (99%), boric acid (99.5 %), and borax (99%) were all of  
26 analytical grade, and obtained from Sigma-Aldrich. Benzene-1,3,5-tricarbaldehyde  
27 (BTA, purity 98%) was purchased from Manchester Organics. *O*-carboxymethyl  
28 chitosan (CMCS) with degree of carboxymethylation of 80 %, degree of deacetylation  
29 of 90 % was supplied by Golden-shell Biochemical Co., Ltd. TP5 (99%) was purchased  
30 from Soho-Yiming Pharmaceutical Co., LTD (Shanghai, China). All chemicals were



1 used as received without further purification.

2

### 3 2.2 Synthesis of dynamer

4 A dynamer (Dy) was synthesized from Jeffamine and BTA, as reported in our previous  
5 work [33]. Typically, BTA (81 mg, 0.5 mmol) and Jeffamine (0.95 g, 0.5 mmol) were  
6 dissolved in 20 mL methanol. The mixture was then heated to 70 °C and allowed to  
7 react for 4 h under gentle stirring. The solvent was totally removed by using rotary  
8 evaporator. 10 mL Milli Q water was added to yield a homogeneous Dy solution at a  
9 concentration of 0.05 M, as calculated from the remaining aldehyde groups.

10

### 11 2.3 Depolymerization of CMCS

12 Low molar mass CMCS was prepared via degradation of original CMCS in the presence  
13 of H<sub>2</sub>O<sub>2</sub> [36]. Typically, CMCS (1 g) was dissolved in 30 mL Milli-Q water under  
14 stirring at 25 °C. 0.15 mL H<sub>2</sub>O<sub>2</sub> was then dropped in the solution. After 30 min  
15 degradation, the solution was immediately frozen by immersing in liquid nitrogen, and  
16 transferred onto a freeze dryer. After 24 h lyophilization, a white powder was obtained.  
17 The product was dissolved in 10 mL Milli-Q water, followed by precipitation in 200  
18 mL absolute ethanol. Finally, the product was obtained by vacuum drying at 40°C for  
19 24 h. Another product was obtained after 60 min degradation using the same process.  
20 The M<sub>n</sub> of original CMCS and depolymerized ones for 30 and 60 min are 80 000,  
21 30 000 and 25 000, respectively, as determined by GPC in aqueous medium. They are  
22 accordingly named as CMCS80K, CMCS30K and CMCS25K.

23

### 24 2.4 Preparation of dynamic hydrogels

25 Dynamic hydrogels were prepared from CMCS, Jeffamine and BTA as previously  
26 reported [33, 34]. Typically, CMCS solution (0.15 M) was prepared by dissolving 0.318  
27 g CMCS (1.5 mmol, calculated from D-glucosamine units) in 10 mL Milli-Q water at  
28 room temperature. The CMCS solution (0.15 M) was then mixed with the Dy solution

1 (0.05 M) with a 4/1 molar ratio of glucosamine/Dy. After removal of trapped bubbles  
2 by using ultra-sound, gelation was allowed to proceed at 37 °C for 24 h to yield a  
3 transparent hydrogel named as Gel80K, Gel30K or Gel25K according to the  $M_n$  of  
4 CMCS.

## 5 6 2.5 Preparation of TP5 loaded hydrogels

7 TP5 loaded hydrogels were prepared through *in situ* encapsulation. In brief, 2.625 mg  
8 TP5 was dissolved in 0.5 mL CMCS80K (0.15 M). The resulting solution was mixed  
9 with 0.375 mL Dy solution (0.05 M). The glucosamine of CMCS to Dy molar ratio was  
10 4/1. After removal of bubbles, gelation then proceeded at 37 °C for 24 h to yield a TP5-  
11 loaded hydrogel with TP5 concentration of 3 mg/mL, namely Gel80K-TP5-3. Other  
12 TP5 loaded hydrogels were prepared using the same procedure but changing one  
13 parameter: Gel80K-TP5-0.1, Gel80K-TP5-9, Gel80K-TP5-3 (25°C) whose gelation  
14 occurred at 25°C, and Gel80K-TP5-3B prepared by dissolving TP5 in Dy solution  
15 before mixing with CMCS80K solution. TP5 was also loaded in Gel30K or Gel25K,  
16 and the corresponding samples were named as Gel30K-TP5-3 or Gel25K-TP5-3,  
17 respectively. In situ incorporation of TP5 results in complete drug encapsulation.  
18 Therefore, the drug loading capacity of all the TP5-loaded hydrogels is 100 %.

19

## 20 2.6 Characterization

21 <sup>1</sup>H NMR spectroscopy was carried out using Bruker NMR spectrometer (400-LS) at  
22 400 MHz. D<sub>2</sub>O was used as the solvent. 5 mg of sample were dissolved in 0.5 mL of  
23 solvent for each analysis.

24 Gel permeation chromatography (GPC) was performed on a Water 1515 multidetector  
25 GPC system equipped with Waters 410 differential detector and ultra-hydrogel 250  
26 column. 0.1 M NaCl was used as the mobile phase with a flow velocity of 0.6 mL/min.  
27 The column temperature was set at 40 °C, and the sample concentration was 5.0 mg/mL.  
28 Poly(ethylene glycol) standards were used for calibration.

1 Fourier-transform infrared (FT-IR) spectroscopy of freeze-dried hydrogels was  
2 conducted on Nicolet Nexus FT-IR spectrometer, equipped with ATR diamant Golden  
3 Gate.

4 The morphology of freeze-dried hydrogels was examined by using Hitachi S4800  
5 scanning electron microscopy (SEM). As-prepared hydrogels were placed in small  
6 vials, and immersed in liquid nitrogen (-196 °C) in order to conserve the original  
7 structure. The frozen samples were lyophilized with LABCONCO® freeze dryer for 24  
8 h, and sputter coated prior to analysis.

9 Physical MCR 301 Rheometer (Anton Paar) was utilized to determine the rheological  
10 properties of hydrogels, using a cone plate geometry (diameter of 4 cm, apex angle of  
11 2°, and clearance of 56 µm). The storage modulus ( $G'$ ) and loss modulus ( $G''$ ) were  
12 measured as a function of time, strain or frequency.

13

#### 14 2.7 Swelling of freeze-dried hydrogels

15 The swelling performance of freeze-dried gels was evaluated by immersion in buffer  
16 solutions at different pH values. Buffers from pH 3 to pH 7.4 were prepared from 0.1  
17 M citric acid and 0.2 M disodium hydrogen phosphate dodecahydrate solutions, buffers  
18 at pH 8 and pH 9 from 0.2 M boric acid and 0.05 M borax solutions, and buffer at pH  
19 10 from 0.05 M borax and 0.2 M sodium hydroxide solutions. Freeze-dried gel samples  
20 were immersed in a buffer solution, and taken out after 24 h. The swollen hydrogels  
21 were weighed after wiping surface water with filter paper, and weighed again after 24  
22 h lyophilization. The swelling and mass loss ratios of hydrogels were calculated  
23 according to the following equations:

$$24 \text{ Swelling ratio } \% = \frac{(M_s - M_d)}{M_d} \times 100 \quad (\text{Eq. 1})$$

$$25 \text{ Mass loss ratio } \% = \frac{(M_0 - M_d)}{M_0} \times 100 \quad (\text{Eq. 2})$$

26 Where  $M_0$  is the initial mass of dried gel,  $M_s$  is the wet mass of the swollen hydrogel,  
27 and  $M_d$  is the dried mass of the swollen hydrogel after lyophilization.

28

## 1 2.8 *In vitro* release of TP5

2 *In vitro* drug release was realized in pH = 7.4 phosphate buffered saline (PBS) at 37 °C.  
3 TP5-loaded hydrogel samples were placed in a centrifuge tube (50 mL) containing 10  
4 mL pH 7.4 PBS. *In vitro* release was performed in shaking water bath at 37 °C. At preset  
5 time intervals, 3 mL of the buffer solution were taken out, and replaced by 3 mL fresh  
6 buffer. TP5 concentration of the release supernatant was determined by HPLC-UV. A  
7 calibration curve was previously established using TP5 solutions with known  
8 concentrations. *In vitro* release of TP5 was also conducted in acidic buffer (pH 5.5),  
9 using the same procedure.

10 The cumulative release of TP5 was calculated using equation 3:

$$11 \text{ Release rate} = \frac{V_e \sum_{i=1}^{n-1} C_i + V_0 C_n}{M_{drug}} \times 100 \quad (\text{Eq. 3})$$

12 Where  $C_n$  and  $C_i$  represent the drug concentrations in the supernatant for  $n$  and  $i$   
13 withdrawing steps, respectively;  $V_0$  is initial total volume of PBS;  $V_e$  is collected  
14 volume of PBS;  $M_{drug}$  is the original loading amount of TP5. The drug loading  
15 efficiency is 100 % due to the *in situ* encapsulation of TP5. All of the experiments were  
16 performed in triplicate, and values were given in mean  $\pm$  SD for  $n = 3$ .

17

## 18 2.9 The release kinetics model

19 The release kinetics of the various TP5-loaded hydrogel systems were studied by fitting  
20 the *in vitro* release data using Higuchi's model [37, 38]:

$$21 Q_t = K_H \times t^{\frac{1}{2}} \quad (\text{Eq. 4})$$

22 where  $Q_t$  is the quantity of drug released within the time  $t$ ,  $K_H$  is the Higuchi dissolution  
23 constant.

24

## 25 2.10 Density functional theory (DFT) computation

26 All calculations were performed using the Dmol<sup>3</sup> code in the Materials Studio 7.0  
27 package [39]. The GGA/PBE functional was selected, and double numerical plus  
28 polarization (DNP) was used as the basis set [40]. Convergence tolerances for the total

1 energy, maximum force, and maximum displacement were set to  $1.0 \times 10^{-5}$  Hartree,  $2.0$   
2  $\times 10^{-3}$  Hartree/Å, and  $5.0 \times 10^{-3}$  Hartree/Å, respectively. The self-consistent field  
3 convergence was  $1.0 \times 10^{-6}$  Hartree. To accelerate the self-consistent field (SCF)  
4 convergence, the smearing was set to 0.005 Hartree. The solvent effect was simulated  
5 by conductor-like screening model (COSMO) [41], using the dielectric constant of  
6 78.54 (water). The transition states were calculated by using the synchronous method  
7 with conjugated gradient refinements. This method involves linear synchronous transit  
8 (LST) maximization, followed by repeated conjugated gradient (CG) minimizations,  
9 then quadratic synchronous transit (QST) maximizations and repeated CG  
10 minimizations until a transition state is located [42]. The energy barrier ( $E_{Barrier}$ ) was  
11 calculated using equation 5:

$$12 \quad E_{Barrier} = E_{TS} - E_{IN} \quad (\text{Eq. 5})$$

13 where  $E_{TS}$  is the energy of the transition state, and  $E_{IN}$  the energy of the intermediate  
14 connected to the transition state.

15 The reaction energy ( $E_{RE}$ ) of the elementary steps was calculated using equation 6:

$$16 \quad E_{RE} = E_P - E_R \quad (\text{Eq. 6})$$

17 where  $E_P$  is the energy of the elementary reaction product, and  $E_R$  the initial energy  
18 of the elementary reaction reactant.

19

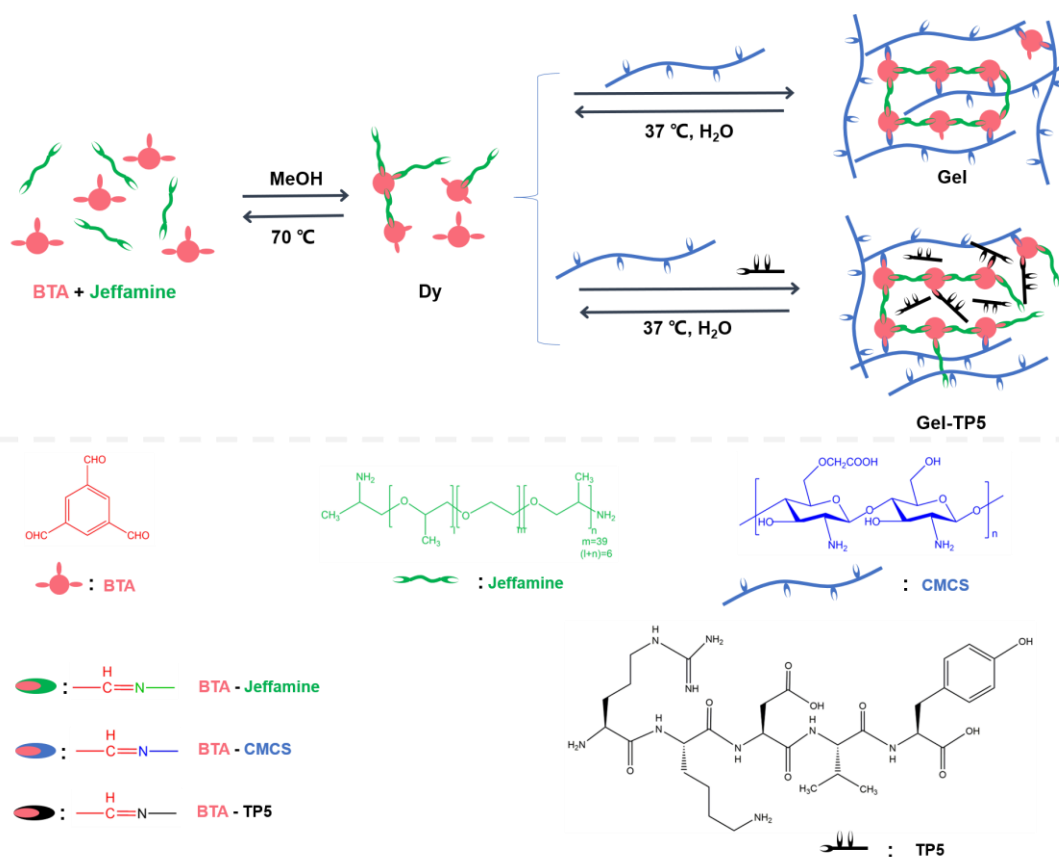
### 20 **3. Results and discussion**

#### 21 **3.1 Preparation of hydrogels**

22 Dynamic hydrogels were synthesized via Schiff-base reaction between CMCS and the  
23 dynamer, as shown in Scheme 1. Upon mixing CMCS and Dy aqueous solutions, the  
24 amino groups along the CMCS backbone react with the remaining aldehyde groups of  
25 Dy, resulting in formation of a 3D hydrogel network by dual imine bonding. The CMCS  
26 to Dy molar ratio of 4:1 was adopted since it allows optimal crosslinking, as  
27 demonstrated in our previous work [33]. In situ TP5 encapsulation in hydrogels is also  
28 shown in Scheme 1. Table 1 summarizes all the details for the preparation of blank and

1 drug loaded hydrogels.

2



3

4 **Scheme 1.** Synthesis of dynamic hydrogels via Schiff-base reaction between CMCS

5 and Dy, and in-situ loading of TP5 in hydrogels.

6

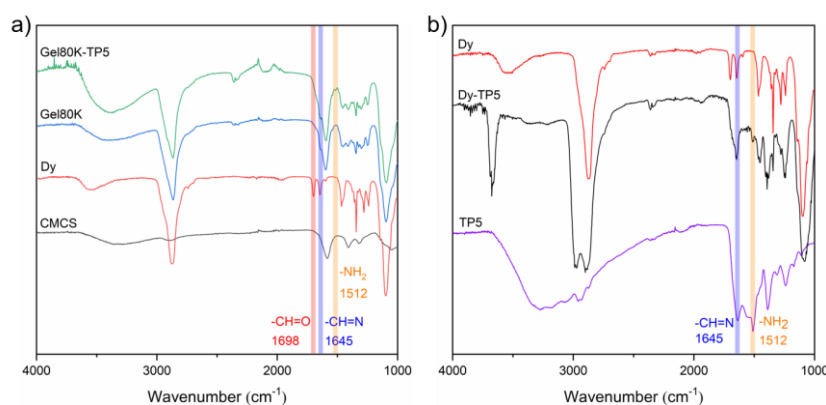
7 **Table 1.** Molar and mass compositions of blank and TP5 loaded hydrogels <sup>a)</sup>

Sample	CMCS80K		CMCS30K		CMCS25K		Dy <sup>c)</sup>		TP5 <sup>d)</sup> [mg/mL]
	[mmol]	[w/v%]	[mmol]	[w/v%]	[mmol]	[w/v%]	[mmol]	[w/v%]	
	b)		b)		b)				
Gel80K	0.3	1.8	-	-	-	-	0.075	4.3	-
Gel30K	-	-	0.3	1.8	-	-	0.075	4.3	-
Gel25K	-	-	-	-	0.3	1.8	0.075	4.3	-
Gel80K-TP5- 0.1	0.3	1.8	-	-	-	-	0.075	4.3	0.1

Gel80K-TP5-3	0.3	1.8	-	-	-	-	0.075	4.3	3
Gel80K-TP5-3B <sup>d)</sup>	0.3	1.8	-	-	-	-	0.075	4.3	3
Gel80K-TP5-3 (25 °C) <sup>e)</sup>	0.3	1.8	-	-	-	-	0.075	4.3	3
Gel80K-TP5-9	0.3	1.8	-	-	-	-	0.075	4.3	9
Gel30K-TP5-3	-	-	0.3	1.8	-	-	0.075	4.3	3
Gel25K-TP5-3	-	-	-	-	0.3	1.8-	0.075	4.3	3

1 a) Hydrogels were prepared by mixing the aqueous solutions of 0.15 M CMCS80K/CMCS30K/CMCS25K and 0.05  
2 M Dy at a D-glucosamine/Dy molar ratio of 4/1 (total volume of 3.5 mL), TP5 loaded hydrogels were prepared by  
3 adding TP5 in CMCS solution before mixing with Dy solution; b) Moles of glucosamine units of CMCS.  
4 Calculations were made on the basis of the average molar mass of 212 g/mol obtained for D-glucosamine, taking  
5 into account the degree of deacetylation of 90% and the degree of carboxymethylation of 80%; c) The concentration  
6 of Dy solution was obtained from the remaining aldehyde groups. In a typical reaction, 0.5 mmol BTA (81 mg)  
7 reacted with 0.5 mmol Jeffamine ED2003 (950 mg) to form a Dy solution. As BTA had 3 aldehydes and Jeffamine  
8 2 amines, there remained theoretically 0.5 mmol of aldehydes in the dried Dy. Addition of 10 mL water yielded a  
9 dynamer solution of 0.05 M. d) Gel80K-TP5-3B was prepared by adding TP5 in Dy solution before mixing with  
10 CMCS solution e) Gel80K-TP5-3 (25 °C) was prepared at 25 °C; f) The concentration of TP5 in hydrogel was 0.1  
11 mg/ml or 3 mg/ml or 9 mg/ml.

### 13 3.2 FT-IR and NMR



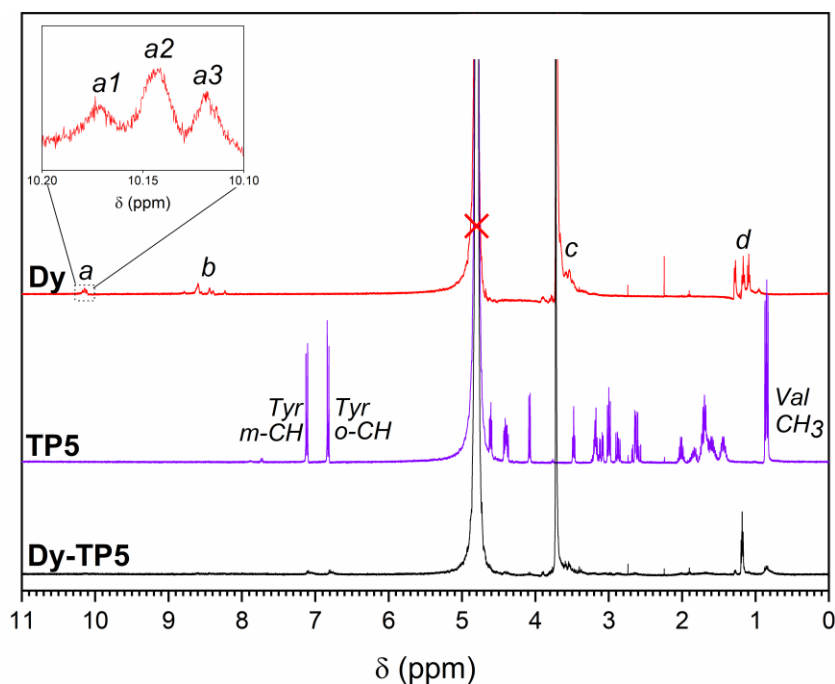
14  
15 **Fig. 1.** a) FT-IR spectra of freeze-dried CMCS, Dy, Gel80K and Gel80K-TP5-3; b) FT-  
16 IR spectra of Dy, TP5, and Dy-TP5 which is an obtained by mixing TP5 in Dy solution  
17 at a molar ratio of 1/1, reaction at 37°C for 24h, and 24 h lyophilization.

18 Fig. 1a shows the FTIR spectra of CMCS, Dy, freeze dried Gel80K and TP5 loaded

1 Gel80K-TP5-3. CMCS presents a large band around  $3350\text{ cm}^{-1}$  which is attributed to  
2 free OH and  $\text{NH}_2$  groups, and an intense band at  $1595\text{ cm}^{-1}$  due to carbonyl groups. The  
3 dynamer exhibits two intense bands at  $2850$  and  $1100\text{ cm}^{-1}$  assigned to C-H and C-O  
4 stretching, and two characteristic bands at  $1698$  and  $1645\text{ cm}^{-1}$  corresponding to  
5 aldehyde and imine bonds, respectively. All the characteristic bands of CMCS and Dy  
6 are observed on the spectrum of freeze dried Gel80K. Nevertheless, the band of  
7 aldehyde groups at  $1698\text{ cm}^{-1}$  is almost invisible, but the band at  $1645\text{ cm}^{-1}$   
8 corresponding to newly formed imine bonds is clearly distinguished. These results  
9 illustrate that hydrogels are formed due to imine formation between aldehydes of Dy  
10 and amine groups of CMCS. On the spectrum of Gel80K-TP5-3, a band is detected at  
11  $2100\text{ cm}^{-1}$ , which is assigned to the O-H stretching of water [43]. In fact, the freeze-  
12 dried samples rapidly absorbed water due to the high hygroscopicity of TP5 [44, 45].  
13 Similar findings are obtained for other blank and TP5 loaded hydrogels (Fig. S1).  
14 Fig. 1b presents the FT-IR spectra of Dy, TP5 and a Dy-TP5 mixture. The latter was  
15 prepared in order to figure out the reactions between the aldehydes of Dy and the amines  
16 of TP5. It was obtained by dissolving TP5 in Dy solution at a molar ratio of 1/1,  
17 followed by 24 h reaction at  $37\text{ }^\circ\text{C}$  and finally 24 h lyophilization. TP5 presents  
18 characteristic bands at  $1630$  and  $1512\text{ cm}^{-1}$  which are assigned to the C=O stretching of  
19 amides and N-H pending of amines, together with a large band around  $3300\text{ cm}^{-1}$  due  
20 to the presence of water. Interestingly, the band of aldehydes at  $1698\text{ cm}^{-1}$  is not detected  
21 on the spectrum of Dy-TP5, suggesting that the aldehydes have reacted with the amine  
22 groups of TP5 via Schiff-base reaction.

23





1

2 **Fig. 2.**  $^1\text{H}$  NMR spectra of the Dy, TP5, and Dy-TP5 in  $\text{D}_2\text{O}$ .

3

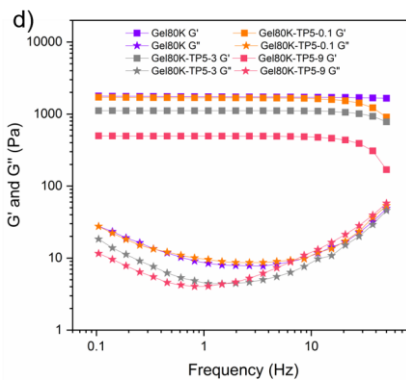
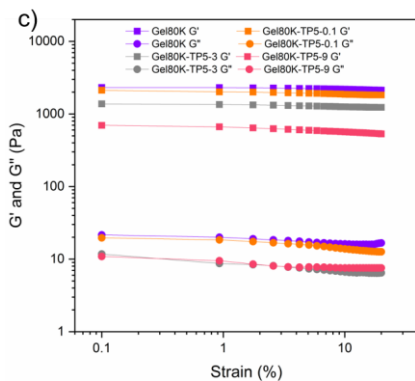
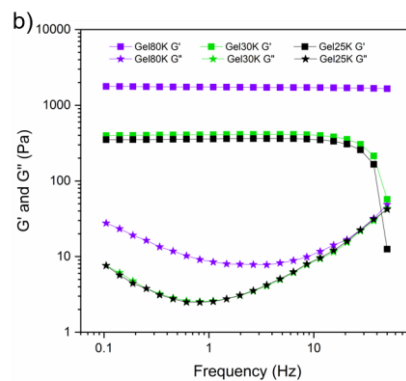
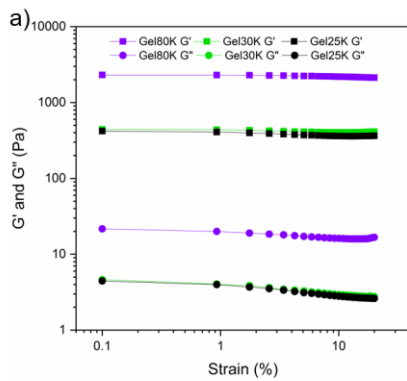
4 Fig. 2 shows the  $^1\text{H}$  NMR spectra of the Dy, TP5, and Dy-TP5. On the spectrum of Dy,  
 5 the three small signals in the 10.1-10.2 ppm range are assigned to aldehyde groups of  
 6 BTA with different degrees of substitution, including non-substituted (a1), mono-  
 7 substituted (a2), and di-substituted aldehydes (a3). Signals between 8.2 and 8.7 ppm  
 8 belong to the aromatic protons of BTA and imine protons (b). Signals around 3.7 ppm  
 9 are assigned to the methylene and methine protons (c) of Jeffamine, and signals between  
 10 1.0 and 1.3 ppm to the methyl protons (d) of Jeffamine [33, 34]. The presence of  
 11 residual  $\text{H}_2\text{O}$  is detected at 4.8 ppm. TP5 presents numerous signals associated to the  
 12 various protons of the pentapeptide (Arg-Lys-Asp-Val-Tyr). The two doublets around  
 13 7.15 and 6.85 ppm are assigned to the meta-CH (Tyr m-CH) and ortho-CH (Tyr o-CH)  
 14 on the phenyl ring of tyrosine [46], respectively. The three signals between 4.0 and 4.7  
 15 ppm belong to the various methine protons, and the signals between 1.3 and 3.6 ppm to  
 16 the various methylene protons of the pentapeptide. The strong signal at 0.85 ppm is  
 17 attributed to the methyl protons of valine (Val  $\text{CH}_3$ ) [46]. Importantly, no signals in the  
 18 range of 10.1-10.2 ppm are observed on the spectrum of Dy-TP5, implying that all the  
 19 remaining aldehydes of Dy have completely reacted with the amine groups of TP5, in

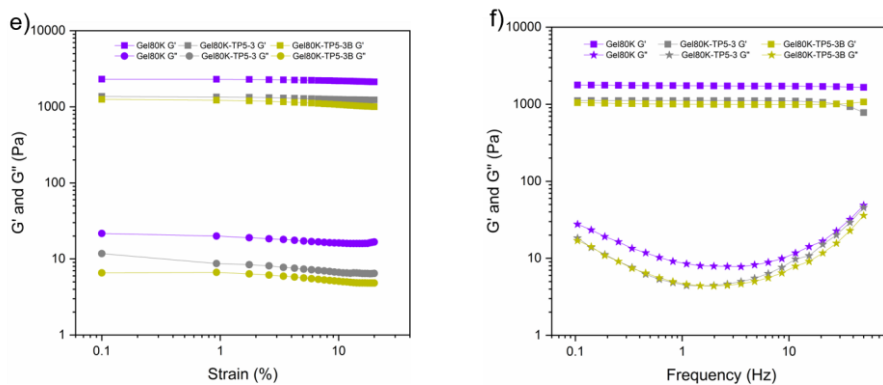
1 agreement with FTIR data (Fig. 1b).

2 The morphology of CMCS, Dy, their mixtures with TP5 (CMCS-TP5 and Dy-TP5) was  
3 examined by using SEM (Fig. S2). No obvious difference is observed between pristine  
4 CMCS (Fig. S2a, b) and CMCS-TP5 (Fig. S2e, f). Both samples display a lamellar  
5 structure with the presence of holes of *c.a* 10  $\mu\text{m}$ , suggesting the absence of interactions  
6 between the two components. In contrast, difference is detected between pristine Dy  
7 (Fig. S2c, d) and the mixture Dy-TP5 (Fig. S2g, h). Both samples exhibit a highly  
8 porous structure. The surface of Dy appears rugged, whereas that of Dy-TP5 appears  
9 smooth. These findings seem to indicate the occurrence of interactions between TP5  
10 and the Dy.

11

### 12 3.3 Rheology





1  
 2 **Fig. 3.** Storage modulus ( $G'$ ) and loss modulus ( $G''$ ) changes of Gel80K, Gel30K and  
 3 Gel25K as a function of strain at 25 °C and at 1 Hz (a) and as a function of frequency  
 4 at 25 °C and at a strain of 1 % (b);  $G'$  and  $G''$  changes of Gel80K, , Gel80K-TP5-0.1,  
 5 Gel80K-TP5-3 and Gel80K-TP5-9 as a function of strain at 25 °C and at 1 Hz (c) and  
 6 as a function of frequency at 25 °C and at a strain of 1% (d);  $G'$  and  $G''$  changes of  
 7 Gel80K, Gel80K-TP5-3, and Gel80K-TP5-3B as a function of strain at 25 °C and at 1  
 8 Hz (e) and as a function of frequency at 25 °C and at a strain of 1 % (f).

9  
 10 Rheological measurements were conducted on the various hydrogels at 25 °C to  
 11 evidence the effect of CMCS molar mass on the rheological properties as well as the  
 12 interaction between TP5 and Dy in the hydrogel network. Firstly, the storage modulus  
 13 ( $G'$ ) and loss modulus ( $G''$ ) changes of blank hydrogels (Gel80K, Gel30K and Gel25K)  
 14 were determined as a function of strain at a frequency of 1 Hz and at 25 °C, as shown  
 15 in Fig. 3a. As the strain increases from 0.1 % to 20 %,  $G'$  remains almost constant for  
 16 all the blank hydrogels, whereas  $G''$  slightly decreases, indicating a linear viscoelastic  
 17 (LVE) behavior of hydrogels in the 0.1 to 20 % strain range. Meanwhile, the moduli of  
 18 blank hydrogels are highly dependent on the molar mass of CMCS. With the decrease  
 19 of molar mass of CMCS from 80,000 to 30,000 and to 25,000,  $G'$  at a strain of 1%  
 20 decreases from 2300 Pa for Gel80K to 440 Pa for Gel30K, and to 420 Pa for Gel25K,  
 21 respectively. Similar phenomena are observed for  $G''$  changes. In fact, higher molar  
 22 mass chains have more amino groups, which allows to enhance the crosslinking density,  
 23 thus resulting in higher modulus of hydrogels

1 Fig. 3b shows the modulus changes of blank hydrogels as a function of frequency from  
2 0.1 Hz to 50 Hz at a constant strain (1%) and at 25 °C.  $G'$  remains constant in the whole  
3 frequency range for Gel80K, whereas a sharp decrease of  $G'$  is observed beyond 30 Hz  
4 in the cases of Gel30K and Gel25K. On the other hand,  $G''$  exhibits some fluctuations  
5 with increasing frequency. These rheological results well corroborate with the  
6 formation of highly stable covalent networks, in contrast to physical hydrogels whose  
7 storage and loss moduli increase with increasing frequency due to chain entanglements  
8 [19, 47]. It is also noteworthy that Gel80K exhibit higher stability than Gel30K and  
9 Gel25K due to higher crosslinking density as mentioned above.

10 Fig. 3c presents  $G'$  and  $G''$  changes *versus* strain of Gel80K hydrogels containing  
11 various amounts of TP5 (0, 0.1, 3, and 9 mg/mL). Obviously, with addition of TP5 in  
12 hydrogels,  $G'$  decreases from 2310 Pa for Gel80K to 2222 Pa for Gel80K-TP5-0.1, to  
13 1374 Pa for Gel80K-TP5-3, and to 697 Pa for Gel80K-TP5-9. These results illustrate  
14 that *in situ* TP5 encapsulation affects the rheological properties of drug loaded  
15 hydrogels. In fact, the amine groups of TP5 can react with the free aldehyde groups of  
16 Dy, as shown in Fig. 1b and Fig. 2. Hence, there is competition between TP5 and CMCS  
17 after mixing aqueous solution of Dy with that of CMCS and TP5, leading to decreased  
18 imine bonding between CMCS and Dy. On the other hand, attachment of TP5 to the  
19 network does not contribute to the crosslinking because TP5 is a small molecule  
20 compared to CMCS. Therefore, the crosslinking density decreases with increase of TP5  
21 content, and consequently the modulus of hydrogels decreases. A frequency sweep of  
22 hydrogels was performed over a range from 0.01 to 50 Hz at a fixed strain of 1 %.  $G'$   
23 remains constant for Gel80K, and slightly decreases beyond 30 Hz for TP5 loaded  
24 hydrogels, indicating that TP5 encapsulation affects the stability of hydrogels.

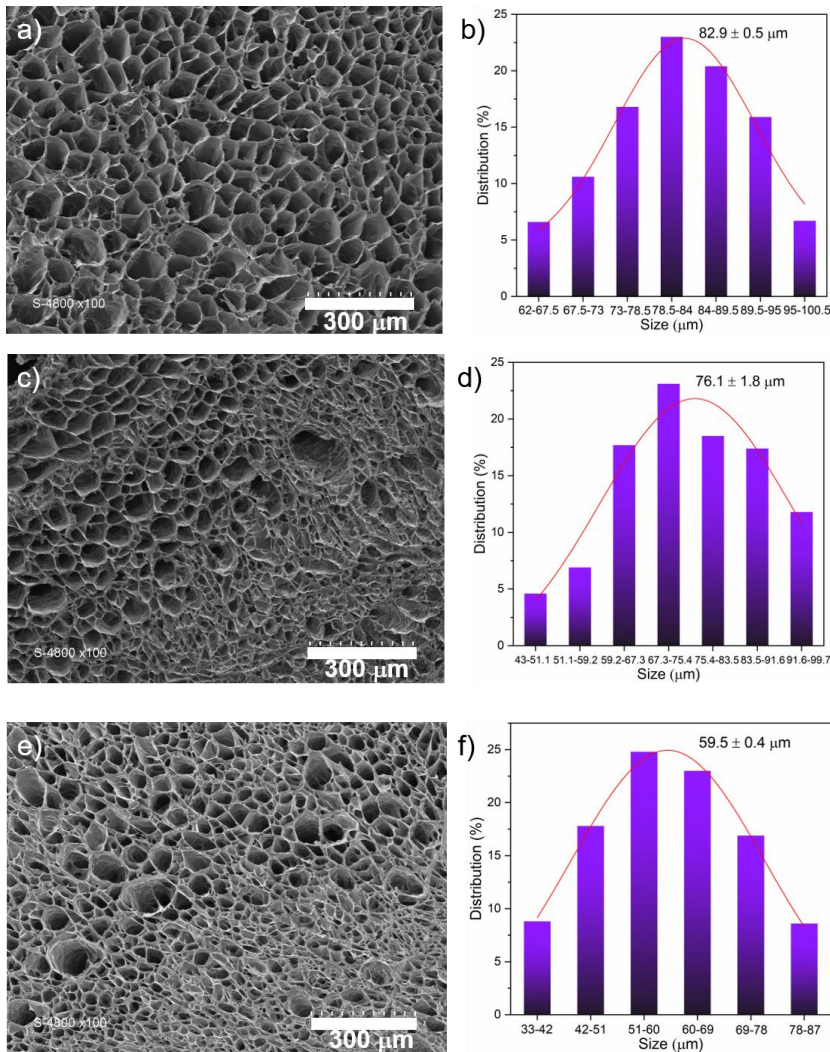
25 A hydrogel (Gel80K-TP5-3B) was prepared by dissolving TP5 in Dy solution,  
26 followed by mixing with CMCS solution before gelation. As TP5 is supposed to react  
27 with Dy, dissolving TP5 in Dy solution should favor imine formation between them.  $G'$   
28 and  $G''$  changes of Gel80K, Gel80K-TP5-3, and Gel80K-TP5-3B were examined as a

1 function of strain and frequency (Fig. 3e, f). Noticeably,  $G'$  of Gel80K-TP5-3 is higher  
 2 than that of Gel80K-TP5-3B in nearly the whole strain and frequency ranges, which  
 3 confirms that mixing TP5 and Dy first decreased the crosslinking density of hydrogels.  
 4 Strain and frequency sweeps were also performed on blank and TP5 loaded Gel30K  
 5 and Gel25K hydrogels (Fig. S3). Similar phenomena were observed, illustrating the  
 6 stability of hydrogels and interactions between TP5 and the dynamer.

7

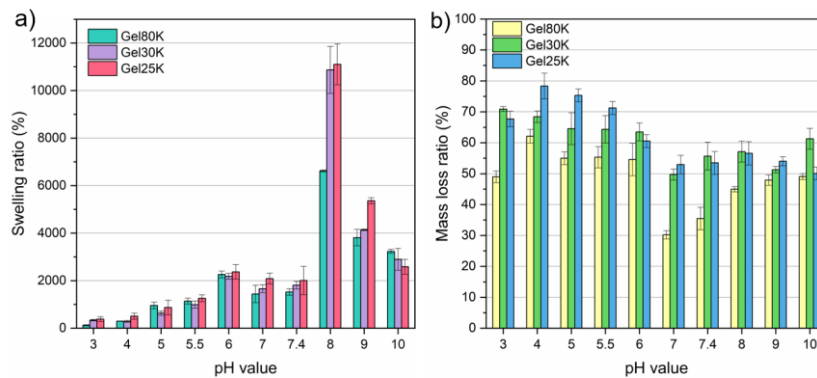
### 8 3.4 Morphology and swelling properties

9



13 **Fig. 4.** SEM images and pore size distributions of freeze-dried gels: (a, b) Gel80K; (c,  
 14 d) Gel30K; (e, f) Gel25K.

1 The morphology of as-prepared hydrogels after lyophilization was observed by SEM.  
2 Obviously, all hydrogels exhibit interpenetrating and porous architectures. Compared  
3 to Gel30K and Gel25K, Gel80K displays more regular structure and narrower  
4 distribution of interconnected pores (Fig. 4a). The average pore diameter of Gel80K,  
5 Gel30K and Gel25K is  $82.9 \pm 0.5 \mu\text{m}$  (Fig. 4b),  $76.1 \pm 1.8 \mu\text{m}$  (Fig. 4d), and  $59.5 \pm 0.4$   
6  $\mu\text{m}$  (Fig. 4f), respectively. Thus, it seems that the pore diameter slightly decreases with  
7 decrease of the molar mass of CMCS. Moreover, in a previous work, it was shown that  
8 the pore diameter of dynamic hydrogels is dependent on the molar mass of Jeffamine  
9 linker [34]. Therefore, the porous structure of dynamic hydrogels can be tailored by  
10 varying the molar mass of the components. On the other hand, the interconnected  
11 porous structure enables hydrogels to mimic the extracellular matrix, which is  
12 beneficial for biomedical applications in drug delivery [48], and in tissue engineering  
13 [33, 49]. Therefore, Gel80K which has the optimal porous “spongy” characteristics was  
14 selected as the carrier of an in-depth study of drug release.  
15



16  
17 **Fig. 5.** a) Swelling ratios, and b) Mass loss ratios of Gel80K, Gel30K and Gel25K after  
18 24 h immersion in buffers at various pH values. Data are expressed as mean  $\pm$  SD for n  
19 = 3.

20 As aforementioned, the most important characteristic of hydrogels is their capability to  
21 retain large amount of water in the polymeric network, which is beneficial for  
22 controlling the release profiles of drugs and the absorption of wound exudate [50]. Fig.  
23 5a presents the swelling performance of freeze-dried hydrogels in buffers at various pH

1 values. All the hydrogels exhibit a highly pH-dependent swelling behavior. The  
2 swelling degree is relatively low ( $< 1000\%$ ) in strong acid buffers (pH 3/4), and  
3 increases to around  $2000\%$  from pH 4 to 6. It remains at the same level around  $2000\%$   
4 in the pH 6 to 7.4 range. All hydrogels exhibit a maximum swelling at pH 8,  $6600\%$   
5 for Gel80K,  $10000\%$  for Gel30K, and  $11000\%$  for Gel25K. As the alkalinity continues  
6 to increase to pH 9 and 10, the swelling degree of hydrogel decreases dramatically to a  
7 level around  $3000\%$ . In addition, there is no major difference between the swelling  
8 ratios of the three samples except at pH 8. The pH sensitivity of the swelling of  
9 hydrogels is assigned to electrostatic interactions between the amino and carboxyl  
10 groups along CMCS chains [33, 51]. In fact, the  $pK_a$  of amino and carboxyl groups of  
11 CMCS is 6.5 and 2.7, respectively. Contraction of hydrogels occurs because of strong  
12 electrostatic attraction between negatively charged  $-\text{COO}^-$  and positively charged  $-\text{NH}_3^+$   
13 groups which result from protonation in strong acidic media (pH 3 and 4), thus leading  
14 to low swelling ratio. Electrostatic attraction diminishes with the decrease of the  $\text{H}^+$   
15 concentration (pH 5, 5.5, and 6), and thus the swelling gradually increases. At pH 6, 7  
16 and 7.4 which are around the  $pK_a$  of amino groups, the swelling remains almost constant  
17 as the electrostatic interactions are weak due to low concentrations of charges. In  
18 contrast, high swelling of hydrogels occurs because of strong repulsion between  
19 negatively charged  $-\text{COO}^-$  groups at pH 8. Nevertheless, with higher alkalinity at pH 9  
20 and 10,  $\text{OH}^-$  ions in solution would counterbalance the electrostatic repulsion between  
21  $-\text{COO}^-$  groups [33], thus resulting in decrease of the swelling degree. The pH sensitive  
22 swelling of hydrogels could be of great importance for the design of drug delivery  
23 systems due to large variation of physiological pH at various body sites in normal and  
24 pathological conditions [52].

25 Mass loss of hydrogels occurs during the swelling process due to the solubilization and  
26 washing away of non-crosslinked species, as shown in Fig. 5b. Overall, the mass loss  
27 ratio of all the hydrogels varies between  $30\%$  and  $80\%$  in the whole pH range. The  
28 hydrogels show higher mass loss in acid conditions than in neutral and alkaline

1 conditions. Noticeably, the mass loss ratio of Gel80K is lower than those of Gel30K  
2 and Gel25K in the whole pH range. This finding could be attributed to the better cross-  
3 linking Gel80K as compared to Gel30K and Gel25K because Gel80K was prepared  
4 from CMCS80K with higher molar mass, in agreement with rheological data. The  
5 lowest mass loss ratio is 30 and 36 % obtained for Gel80K at pH 7 and 7.4, respectively,  
6 in agreement with the better stability of Gel80K at neutral pH. Thus, Gel80K is used  
7 for a comprehensive study of TP5 release from hydrogels under various conditions.  
8 The swelling and mass loss behaviors of Gel80K were investigated under drug release  
9 conditions in pH 7.4 PBS at 37 °C up to 168 h (Fig. S4, Information supplementary).  
10 The swelling ratio rapidly increases to 750 % and 1200 % after immersion for 10 and  
11 20 min, respectively, whereas the mass loss ratio increases 25 % and 32 % in the  
12 meantime. Thereafter, the swelling and mass loss ratios remain almost at the same level  
13 up to 168 h, fluctuating around 1300 % and 34 %, respectively. These findings indicate  
14 that an equilibrium is rapidly reached after immersion of dried gels in PBS, and that the  
15 hydrogels are stable after initial mass loss.

16

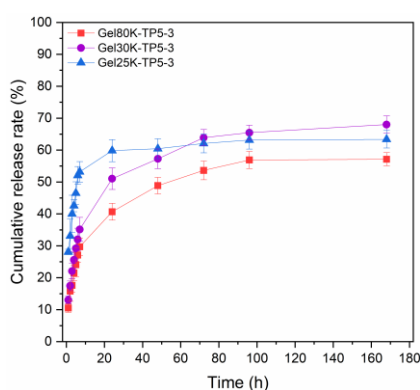
### 17 **3.5 *In vitro* release of the TP5**

18 TP5 is an immuno-modulating drug, and is widely applied in the treatment of  
19 autoimmune diseases such as chronic lymphocytic leukemia [10], Sezary's syndrome  
20 [53, 54], etc. Besides, TP-5 is able to induce the differentiation of T-cells and accelerate  
21 the development of T lymphocytes [11]. A series of TP5-loaded hydrogels were  
22 prepared, including Gel80K-TP5-0.1, Gel80K-TP5-3, Gel80K-TP5-9, Gel80K-TP5-  
23 3B, Gel80K-TP5-3(25°C), Gel30K-TP5-3, and Gel25K-TP5-3 so as to evaluate the  
24 effects of various factors.

25 The morphology of the various TP5-loaded hydrogels was examined by using SEM  
26 (Fig. S5). It appears that TP5 encapsulation affects the regularity of the porous structure  
27 of hydrogels. In fact, Gel80K-TP5-0.1 exhibits a porous structure similar to that of  
28 Gel80K (Fig. 4a,b), but the surface appears rougher. With increase of TP5 content from



1 0.1 mg/mL (Fig. S5a, b) to 3 mg/mL (Fig. S5c, d), and to 9 mg/mL (Fig. S5e, f), the  
2 three-dimensional architecture becomes more and more irregular as compared with that  
3 of Gel80K (Fig. 4a,b). Similarly, TP5 encapsulation also affects the structure of  
4 Gel30K-TP5-3 (Fig. S5i, j) and Gel25K-TP5-3 (Fig. S5k, l) as compared to  
5 corresponding blank hydrogels. On the other hand, difference is also noticed between  
6 the morphology of Gel80K-TP5-3 and Gel80K-TP5-3B (Fig. S5g, h). Both have  
7 exactly the same compositions, but different mixing orders. Gel80K-TP5-3B exhibits a  
8 smoother surface compared to Gel80K-TP5-3.

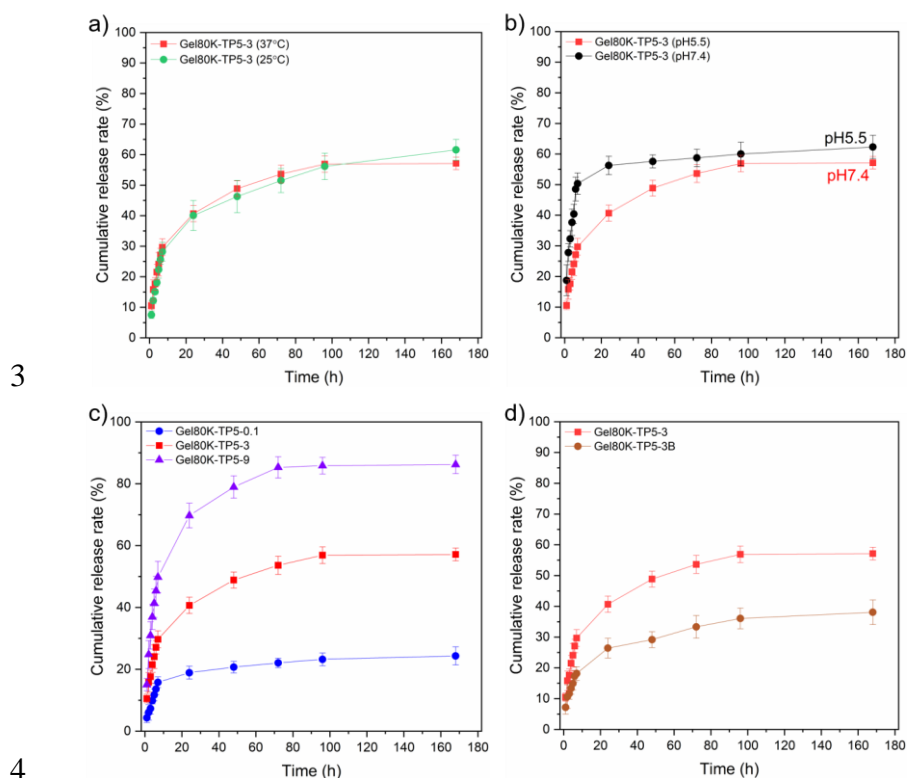


9  
10 **Fig. 6.** Cumulative release of TP5 from Gel80K-TP5-3, Gel30K-TP5-3, and Gel25K-  
11 TP5-3. Values were expressed as mean  $\pm$  SD for  $n = 3$ .

12

13 TP5 release from the various TP5-loaded hydrogels was monitored to evaluate the  
14 potential of dynamic hydrogels as drug carrier in cancer therapy [38]. Fig. 6 shows the  
15 release profiles of TP5 from Gel80K-TP5-3, Gel30K-TP5-3, and Gel25K-TP5-3 in pH  
16 7.4 PBS at 37 °C. Overall, all samples displayed a release profile in two stages, namely  
17 a burst release in the first 7 h followed by a slow and continuous release during 7 days.  
18 After 1 h in the medium, significant release rates are recorded, 28.0% for Gel25K-TP5,  
19 13.0 % for Gel30K-TP5 and 10.5 % for Gel80K-TP5. TP5 release increases almost  
20 linearly to reach 53.2 %, 35.1 % and 29.7 % after 7 h for the 3 samples, respectively.  
21 Thereafter, TP5 release slows down, reaching a plateau after 96 h. The total TP5 release  
22 is 63.4, 68.0, and 57.1% after 168 h for Gel25K-TP5, Gel30K-TP5 and Gel80K-TP5,  
23 respectively. Gel80K-TP5 exhibits slower and more sustainable release as compared to

1 Gel25K-TP5 and Gel30K-TP5 due to its higher crosslinking density which disfavors  
2 drug diffusion. Therefore, Gel80K is used as drug carrier in further studies.



6 **Fig. 7.** a) Cumulative release of TP5 from Gel80K-TP5-3 prepared at 25 °C and 37 °C,  
7 namely Gel80K-TP5-3 (25 °C) and Gel80K-TP5-3 (37 °C); b) Cumulative release of  
8 TP5 from Gel80K-TP5-3 in PBS at pH 7.4, and in acid buffer medium at pH 5.5, namely  
9 Gel80K-TP5-3 (pH 7.4) and Gel80K-TP5-3 (pH 5.5); c) Cumulative release of TP5  
10 from Gel80K loaded with various TP5 contents: Gel80K-TP5-0.1, Gel80K-TP5-3, and  
11 Gel80K-TP5-9; d) Cumulative release of TP5 from Gel80K-TP5-3 and Gel80K-TP5-  
12 3B with different mixing orders. Values were expressed as mean  $\pm$  SD for n = 3.

13

14 Fig. 7 show the TP5 release curves from various hydrogels. No difference is noticed  
15 between the release profiles Gel80K-TP5-3 prepared at 25 °C and at 37 °C (Fig. 7a),  
16 indicating the gelation temperature (25 or 37 °C) of Gel80K-TP5 has little impact on  
17 the release of TP5. In contrast, faster initial release is observed from Gel80K-TP5-3 at  
18 pH 5.5 than at pH 7.4, although both present similar equilibrium release ratios around

1 60% (Fig. 7b). This difference can be assigned to the fact that the imine bonds are prone  
2 to hydrolysis under acidic condition [55], which leads to partial disassociation of the  
3 three-dimensional hydrogel network thus favoring the diffusion of trapped TP5. It is  
4 also of interest to evaluate the effect of initial drug load on the release rate by comparing  
5 the release profiles of Gel80K-TP5-0.1, Gel80K-TP5-3, Gel80K-TP5-9, as shown in  
6 Fig. 7c. Apparently, the latter shows the highest equilibrium rate, reaching 90 % after  
7 168 h, which is much higher than that of Gel80K-TP5-0.1 (22.5%) and Gel80K-TP5-3  
8 (57 %). This difference well corroborates with the partial attachment of TP5 to the  
9 hydrogel network by imine bonding. It can be assumed that only free TP5 is released  
10 as imine bonds are stable at pH 7.4. Thus, hydrogels with higher drug loading have  
11 higher proportion of free drug, and consequently present higher drug release ratio [38].  
12 Finally, TP5 release behaviors from Gel80K-TP5-3 and Gel80K-TP5-3B are compared  
13 to examine the effect of the mixing order (Fig. 7d). Gel80K-TP5-3B presents a total  
14 release ratio of 38%, which is much lower than that of Gel80K-TP5-3 (60%). This  
15 finding also confirms the covalent attachment of TP5 to the hydrogel network by imine  
16 bonding. As TP5 is first mixed with Dy before mixing with CMCS in the case of  
17 Gel80K-TP5-3B, more TP5 is covalently attached to the network, leading to lower TP5  
18 release as compared to Gel80K-TP5-3. These data indicate that only physically  
19 encapsulated TP5 can be released under in vitro conditions. The release rate of TP5  
20 from hydrogels depends on many factors such as the molar mass of CMCS, the pH of  
21 the medium, the drug load, and the mixing order during hydrogel preparation.

22

### 23 3.6 Drug release kinetics

24 Table 2 Parameters obtained from fitting of experimental data using Higuchi model <sup>a)</sup>

Sample	R <sup>2</sup>	K <sub>H</sub>
Gel80K-TP5-0.1	0.967	6.477
Gel80K-TP5-3	0.990	11.110
Gel80K-TP5-9	0.998	20.897

Gel80K-TP5-3B	0.978	6.502
Gel80K-TP5 (pH5.5)	0.982	19.141
Gel80K-TP5 (25°C)	0.985	12.271
Gel30K-TP5	0.997	13.315
Gel25K-TP5	0.986	16.172

1 a) Experimental data in Fig. 6-7.

2

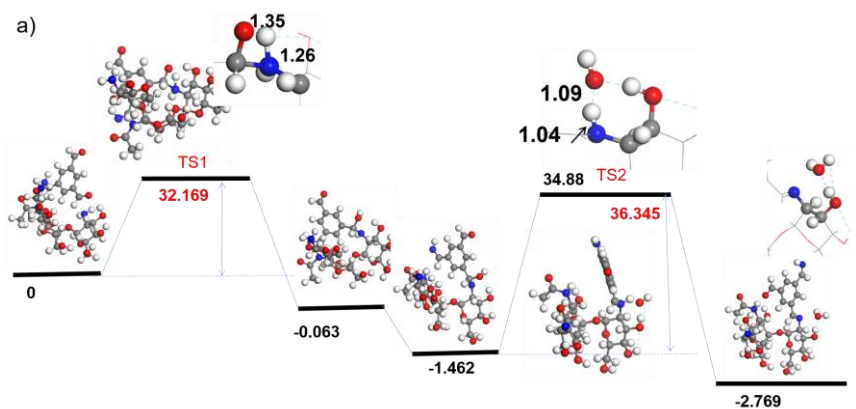
3 It is widely admitted that the release of a hydrophilic drug from a monolithic matrix  
 4 mainly depends on the diffusion process. Other factors such as physical forces between  
 5 the drug and the hydrogel matrix and swelling of bulk hydrogel affect the release profile  
 6 as well. Higuchi's model has been largely used to describe the drug release kinetics of  
 7 various systems. It is based on the Fick's Law where the release proceeds by the  
 8 diffusion of drugs through the delivery system. In this case, the cumulative released  
 9 drug amount is proportional to the square root of time. TP5 release kinetics were  
 10 investigated by fitting of experimental data in the first 6 h using Higuchi model in order  
 11 to better understand the release mechanism of TP5 from dynamic hydrogels [38], as  
 12 shown in Fig. S6 and Table 2.

13 Data in Table 2 show that the correlation coefficient is above 0.98 in all cases except  
 14 Gel80K-TP5-0.1, suggesting that the TP5 release was mainly controlled by diffusion  
 15 process [56]. In fact, the maximum concentration of TP5 after total release in the release  
 16 medium is well below its saturation concentration ( $> 10$  mg/mL) even for Gel80K-  
 17 TP5-9 [19]. Thus, it could be considered that the dissolution is the ruling force of TP5  
 18 release from the hydrogels as drug release was conducted under immersion conditions  
 19 [38]. It is also of interest to compare the  $K_H$  values of the different systems. Gel80K-  
 20 TP5-9 and Gel80K-TP5-0.1 present the highest and the lowest  $K_H$  values, respectively,  
 21 in agreement with the fastest and the slowest release rates. Intermediate  $K_H$  values were  
 22 obtained for other TP5 loaded hydrogels.

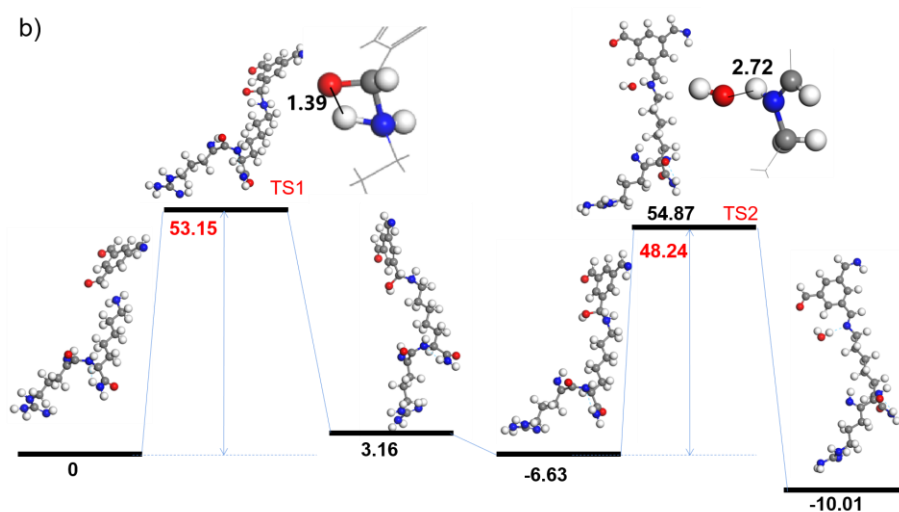
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### 24 **3.7 Density functional theory (DFT) modelling**

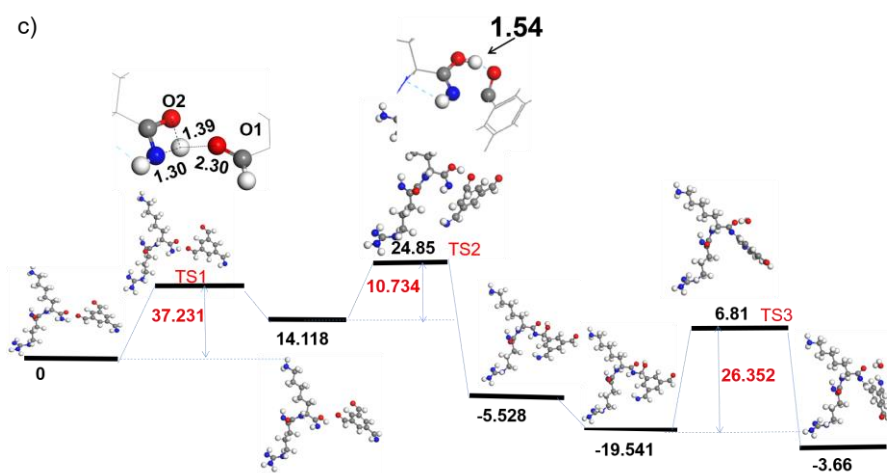
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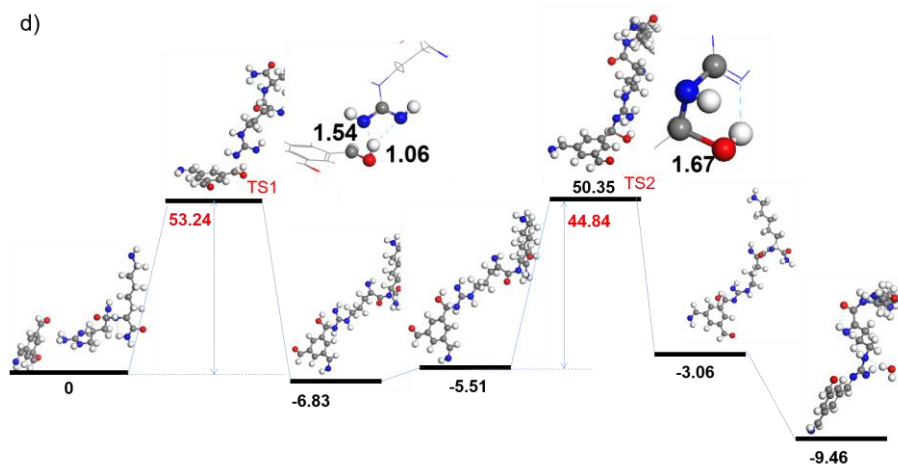
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4



1

2 **Fig. 8.** Computation by density functional theory (DFT) to determine the lowest  
 3 reaction pathways between : a) CMCS-l-Dy; b) TP5(1)-l-Dy (TP5 site 1); c) TP5(2)-  
 4 l-Dy (TP5 site 2); and d) TP5(3)-l-Dy (TP5 site 3). The reaction energy and energy  
 5 barrier are calculated by Eq. 5 and Eq. 6 in kcal/mol.

6

7 Density functional theory (DFT) was used to determine the reaction energy and energy  
 8 barrier of imine linkages between TP5 and Dy (TP5-l-Dy, ‘l’ stands for linkage,  
 9 similarly hereinafter) as well as those between CMCS and Dy (CMCS-l-Dy. The  
 10 simplified models of CMCS, Dy and TP5 are shown in Fig. S7a-c. Only one reaction  
 11 site is considered taking into account the repeatability of the amino groups in CMCS  
 12 and the symmetry of Dy. The lowest energy pathway of the Schiff-base reaction  
 13 between CMCS and Dy is shown in Fig. 8a. The first step of the reaction is the  
 14 dehydrogenation from the amino group of Dy, which requires overcoming an energy  
 15 barrier of 32.2 kcal/mol. The formation of CH-NH bond is thermodynamically  
 16 favorable. This is followed by the cleavage of the oxhydrile group, which needs higher  
 17 energy than the first step, reaching 36.3 kcal/mol. The formation and removing of H<sub>2</sub>O  
 18 molecule are thermodynamically favorable. Thus, the rate-limiting step of the reaction  
 19 between CMCS and Dy is the cleavage of oxhydrile with an energy barrier of 36.3  
 20 kcal/mol.

21 There are three kinds of amino groups in TP5 which have different chemical  
 22 environments (Fig. S7c). Thus, there are three -NH<sub>2</sub> sites that can be involved in the

1 Schiff-base reaction, which are marked as TP5(1)-l-Dy (Fig. 8b), TP5(2)-l-Dy (Fig. 8c)  
2 and TP5(3)-l-Dy (Fig. 8d), respectively. The Schiff-base reactions of TP5(1)-l-Dy and  
3 TP5(3)-l-Dy are similar. The rate-limiting step of both reactions is the dehydrogenation  
4 from the amino groups of TP5 (the first step), which needs to overcome an energy  
5 barrier of 53.15 and 53.24 kcal/mol, respectively. The reaction process to yield TP5(2)-  
6 l-Dy is more complex than those of the two other reactions. It begins with  
7 dehydrogenation from the amino group of TP5. But the H atom tends to bond with the  
8 adjacent oxygen atom (O2), and the process needs to overcome an energy barrier of  
9 37.2 kcal/mol. In the second step, the hydrogen proton transfers from O2 to O1, which  
10 needs an energy of 10.7 kcal/mol. The formation of -C-NH bond is thermodynamically  
11 favorable. Subsequently, the cleavage of oxhydryle needs to overcome a barrier of 26.4  
12 kcal/mol. Thus, the rate-limiting step of the reaction needs to overcome a barrier of 37.2  
13 kcal/mol, which is only 0.9 kcal/mol higher than that of the Schiff-base reaction  
14 between CMCS and Dy. Thus, the -C=O group adjacent to the -NH<sub>2</sub> of TP5 could  
15 promote the Schiff-base reaction between TP5 and Dy to form TP5(2)-l-Dy. Moreover,  
16 the two other -NH<sub>2</sub> sites of TP5 may also participate in the Schiff-base reaction in spite  
17 of higher energy barrier. Hence, although the energy barrier of TP5-l-Dy is higher than  
18 that of CMCS-l-Dy, both reactions proceed concomitantly in a competitive way.  
19 Furthermore, it is worth noting that the gelation time of blank hydrogel without TP5 is  
20 shorter than that of hydrogel containing TP5 (data not shown). Therefore, the presence  
21 of TP5 affects the crosslinking between CMCS and Dy because TP5 reacts  
22 competitively with Dy and formation of TP5-l-Dy does not contribute to the  
23 crosslinking due to steric effect. When TP5 is mixed with Dy, it can theoretically and  
24 experimentally react with Dy via Schiff base reaction, which is in full agreement with  
25 the results obtained by FT-IR (Fig. 1), NMR (Fig. 2), and SEM (Fig. S4), and TP5  
26 release (Fig. 6 and 7).

27

#### 28 **4. Conclusion**

1 This work focused on the synthesis and characterization of a novel drug delivery system  
2 based on dynamic hydrogels for the sustainable release of an immunostimulant –  
3 thymopentin (TP5). The hydrogels were prepared via *in situ* gelation of O-  
4 carboxymethyl chitosan with a dynamer made from Jefamine and BTA in aqueous  
5 solution by imine bonding, in the presence or absence of TP5. These hydrogels display  
6 a porous structure, outstanding rheological properties and excellent pH-sensitive  
7 swelling performance. Analyses showed that TP5 is partly attached to the hydrogel  
8 network by imine bonding, together with free TP5 molecules adsorbed by physical  
9 forces into the hydrogel pore walls. *In vitro* release showed a burst release in the first  
10 24 h, followed by a slower release to reach a plateau up to 168 h. The maximum release  
11 rate depends on various factors such as drug content, drug loading and release  
12 conditions. Data show that only free TP5 is released under *in vitro* conditions.  
13 Covalently attached TP5 is not released due to the stability of imine bonds at pH 7.4.  
14 Density functional theory study allowed to better understand the chemical combination  
15 between the hydrogel's components and the drug. All these findings suggest that *in situ*  
16 gelation of water soluble CMCS with a 3D dynamer is a means of choice to design  
17 efficient release systems of hydrophilic drugs, in particular those containing amino  
18 groups which can be covalently attached to the hydrogel network. Last but not least,  
19 this study offers a simple and environmentally friendly strategy to prepare bioactive  
20 and biodegradable hydrogels for drug delivery systems.

21

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26

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1 **Biobased dynamic hydrogels by reversible imine bonding for**  
2 **controlled release of thymopentin**

3

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15

1 **Abstract**

2 Thymopentin (TP5) is widely used in the treatment of autoimmune diseases, but the  
3 short *in vivo* half-life of TP5 strongly restricts its clinical applications. A series of blank  
4 and TP5 loaded hydrogels were synthesized via reversible dual imine bonding by  
5 mixing water soluble *O*-carboxymethyl chitosan (CMCS) with a dynamer (Dy)  
6 prepared from Jeffamine and benzene-1,3,5-tricarbaldehyde. TP5 release from  
7 hydrogels was studied at 37°C under *in vitro* conditions. The molar mass of CMCS,  
8 drug loading conditions and drug content were varied to elucidate their effects on  
9 hydrogel properties and drug release behaviors. Density functional theory was applied  
10 to theoretically confirm the chemical connections between TP5 or CMCS with Dy. All  
11 hydrogels exhibited interpenetrating porous architecture with average pore size from  
12 59 to 83  $\mu\text{m}$ , and pH-sensitive swelling up to 10 000 % at pH 8. TP5 encapsulation  
13 affected the rheological properties of hydrogels as TP5 was partially attached to the  
14 network via imine bonding. Higher TP5 loading led to higher release rates. Faster  
15 release was observed at pH 5.5 than at pH 7.4 due to lower stability of imine bonds in  
16 acidic media. Fitting of release data using Higuchi model showed that initial TP5  
17 release was essentially diffusion controlled. All these findings proved that the dynamic  
18 hydrogels are promising carriers for controlled delivery of hydrophilic drugs, and shed  
19 new light on the design of drug release systems by both physical mixing and reversible  
20 covalent bonding.

21

22 Keywords: *O*-carboxymethyl chitosan; Imine bonding; Dynamic hydrogel;  
23 Thymopentin; Drug release; Density functional theory

24

## 1 **1. Introduction**

2 Thymopentin (TP5) is a synthetic pentapeptide (Arg-Lys-Asp-Val-Tyr,  $M_n = 679.77$ )  
3 associated with the residual sequence (32-36) of thymic hormone thymopoietin [1, 2].  
4 It possesses the biological properties characteristic of thymopoietin [3], and has been  
5 widely used for immunomodulation [4], and for treatments of autoimmune diseases [5],  
6 including cancer immunodeficiency [6], rheumatoid arthritis [7], tuberculosis [8],  
7 chronic heart failure [9], and chronic lymphocytic leukemia [10]. TP5 can facilitate the  
8 differentiation of thymocytes and affect the function to induce maturation of T-cells [11,  
9 12] and bone marrow dendritic cells [13]. Nevertheless, due to the short half-life ( $\leq 30$   
10 s in plasma) and extensive metabolism in the gastrointestinal tract [14], TP5 is generally  
11 applied as a freeze-dried powder by intramuscular or percutaneous injection [5].  
12 Chronic autoimmune diseases require long therapies and repetitive injections of TP5  
13 which could provoke complications. These disadvantages highly limit the clinical  
14 utilization of TP5.

15 **It has been reported that the in vivo efficiency of TP5 can be improved by slow infusion**  
16 **[15]. Therefore, controlled drug delivery systems are considered as an appealing**  
17 **strategy to achieve slow infusion of TP5**, including nanoparticles [16], micelles [17],  
18 liposomes [18], and hydrogels [19, 20]. These various systems present many  
19 advantages such as prolonged drug release, constant drug concentration, reduced side  
20 effects, protection of drugs from degradation, etc. Micelles, liposomes and  
21 nanoparticles have been used to encapsulate TP5, but the encapsulation efficiency is  
22 quite low because of its high hydrophilicity [17, 21]. Dissolving microneedle array was  
23 studied as a new strategy for TP5 delivery by transdermal injection [4]. However,  
24 customized microneedle arrays and daily administration are needed, leading to high  
25 cost and low bioavailability. In contrast, hydrogel delivery systems appear most  
26 interesting for sustained delivery of hydrophilic drugs, and have been used clinically  
27 [22]. Hydrogels can easily encapsulate a variety of hydrophilic therapeutic agents,  
28 including small-molecule drugs [23], macromolecular drugs [24] and cells [25].  
29 Furthermore, the high-water up taking capacity endows hydrogels with the physical  
30 features similar to tissues, and excellent biocompatibility. Besides, the crosslinked  
31 framework of hydrogels can hinder penetration of proteins [26], and thus protecting  
32 vulnerable bioactive drugs from degradation by inwardly diffusing enzymes [27].

33 Hydrogels have been investigated for the encapsulation of TP5. Zhang et al. developed



1 a physical hydrogel from polylactide-poly(ethylene glycol) (PLA-PEG) block  
2 copolymers via stereo-complexation between poly(L-lactide) and poly(D-lactide)  
3 blocks [19]. High drug loading and rapid drug release were obtained. Nevertheless,  
4 PLA degradation leads to acidic by-products which could provoke inflammatory  
5 reactions, and such physical hydrogels exhibit low stability and mechanical strength.  
6 Su et al. synthesized a hydrogel by crosslinking PLA grafted *O*-carboxymethyl chitosan  
7 (CMCS) using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and *N*-  
8 hydroxysuccinimide (NHS) as crosslinking agents [28]. The resulting hydrogel was  
9 used to encapsulate TP5 by immersion of dried gel in a TP5 solution. More than 80%  
10 of TP5 was released in 48 h. However, the presence of residual chemicals or solvents  
11 could compromise the biocompatibility of the system [29]. Therefore, it becomes a  
12 major challenge to develop a delivery system of TP5 which exhibits good  
13 biocompatibility, stability and high encapsulation efficiency.

14 CMCS is a water-soluble derivative of chitosan, the second most abundant  
15 polysaccharide in nature. It has been studied as a promising carrier for the delivery of  
16 peptides and proteins [30]. On the other hand, Jeffamine is a biocompatible  
17 polyetheramine containing poly(propylene oxide) (PPO) and poly(ethylene oxide)  
18 (PEO) blocks, as well as primary amino groups at chain ends. It has been used in drug  
19 delivery because of its water solubility and cytocompatibility [31]. Recently, our group  
20 has developed a CMCS and Jeffamine based dynamic hydrogel that could be potentially  
21 used for drug delivery [32], cartilage engineering [33], and antibacterial materials [34].  
22 The hydrogel was prepared via dual imine bond crosslinking between the amino groups  
23 of CMCS and the aldehyde ones of a 3D dynamer (Dy). Dy was first obtained by Schiff-  
24 base reaction of 1,3,5-benzenetri-aldehyde (BTA) and di-amino Jeffamine, followed by  
25 Schiff-base reaction of Dy and CMCS in aqueous solution to yield a 3D network.  
26 Trifunctional BTA served as a core to ensure 3D crosslinking, and Jeffamine as a linker  
27 to endow Dy with aqueous solubility. The gelation process is conducted in aqueous  
28 solution under mild conditions, which is beneficial for encapsulation of fragile drugs

1 such as proteins or peptides [22]. Different from other chitosan-based hydrogel by  
2 imine bonding [35], CMCS and Jeffamine based dynamic hydrogels exhibit regular and  
3 interconnected porosity which is beneficial for swelling, diffusion of physiological  
4 fluids, and drug encapsulation [33,34]. Interestingly, the hydrogel network contains free  
5 aldehyde and amino groups which are able to react with the functional groups of drugs  
6 or other bioactive molecules.

7 This work aimed to develop a novel delivery system of TP5 from CMCS and Jeffamine  
8 based dynamic hydrogels by in situ encapsulation. CMCS with different molar masses  
9 of CMCS was used since the molar mass affects the physico-chemical properties of  
10 hydrogels. *In situ* incorporation of TP5 within the dynamic hydrogel should allow to  
11 achieve complete drug encapsulation by physical adhesion to pore walls and reversible  
12 imine bonding to the hydrogel network via Schiff base reaction between the amino  
13 groups of TP5 and aldehydes of the dynamer. The rheological properties, internal  
14 morphology and swelling performance of dynamic hydrogels and TP5-loaded  
15 hydrogels were comparatively investigated. *In vitro* drug release of TP5-loaded  
16 hydrogels was studied under different conditions, and fitted using Higuchi's model.  
17 Finally, density functional theory (DFT) was applied to model the different imine bonds  
18 in the hydrogel network.

19

## 20 2. Experimental section

### 21 2.1 Materials

22 *O,O'*-bis(2-aminopropyl) poly(propylene glycol)-block-poly(ethylene glycol)-block-  
23 poly(propylene glycol), namely Jeffamine<sup>®</sup> ED-2003 with  $M_n$  of 1900, methanol (96%),  
24 hydrogen peroxide solution (H<sub>2</sub>O<sub>2</sub>, 30%) citric acid (99.5%), disodium hydrogen  
25 phosphate dodecahydrate (99%), boric acid (99.5 %), and borax (99%) were all of  
26 analytical grade, and obtained from Sigma-Aldrich. Benzene-1,3,5-tricarbaldehyde  
27 (BTA, purity 98%) was purchased from Manchester Organics. *O*-carboxymethyl  
28 chitosan (CMCS) with degree of carboxymethylation of 80 %, degree of deacetylation  
29 of 90 % was supplied by Golden-shell Biochemical Co., Ltd. TP5 (99%) was purchased  
30 from Soho-Yiming Pharmaceutical Co., LTD (Shanghai, China). All chemicals were

1 used as received without further purification.

2

### 3 2.2 Synthesis of dynamer

4 A dynamer (Dy) was synthesized from Jeffamine and BTA, as reported in our previous  
5 work [33]. Typically, BTA (81 mg, 0.5 mmol) and Jeffamine (0.95 g, 0.5 mmol) were  
6 dissolved in 20 mL methanol. The mixture was then heated to 70 °C and allowed to  
7 react for 4 h under gentle stirring. The solvent was totally removed by using rotary  
8 evaporator. 10 mL Milli Q water was added to yield a homogeneous Dy solution at a  
9 concentration of 0.05 M, as calculated from the remaining aldehyde groups.

10

### 11 2.3 Depolymerization of CMCS

12 Low molar mass CMCS was prepared via degradation of original CMCS in the presence  
13 of H<sub>2</sub>O<sub>2</sub> [36]. Typically, CMCS (1 g) was dissolved in 30 mL Milli-Q water under  
14 stirring at 25 °C. 0.15 mL H<sub>2</sub>O<sub>2</sub> was then dropped in the solution. After 30 min  
15 degradation, the solution was immediately frozen by immersing in liquid nitrogen, and  
16 transferred onto a freeze dryer. After 24 h lyophilization, a white powder was obtained.  
17 The product was dissolved in 10 mL Milli-Q water, followed by precipitation in 200  
18 mL absolute ethanol. Finally, the product was obtained by vacuum drying at 40°C for  
19 24 h. Another product was obtained after 60 min degradation using the same process.  
20 The M<sub>n</sub> of original CMCS and depolymerized ones for 30 and 60 min are 80 000,  
21 30 000 and 25 000, respectively, as determined by GPC in aqueous medium. They are  
22 accordingly named as CMCS80K, CMCS30K and CMCS25K.

23

### 24 2.4 Preparation of dynamic hydrogels

25 Dynamic hydrogels were prepared from CMCS, Jeffamine and BTA as previously  
26 reported [33, 34]. Typically, CMCS solution (0.15 M) was prepared by dissolving 0.318  
27 g CMCS (1.5 mmol, calculated from D-glucosamine units) in 10 mL Milli-Q water at  
28 room temperature. The CMCS solution (0.15 M) was then mixed with the Dy solution

1 (0.05 M) with a 4/1 molar ratio of glucosamine/Dy. After removal of trapped bubbles  
2 by using ultra-sound, gelation was allowed to proceed at 37 °C for 24 h to yield a  
3 transparent hydrogel named as Gel80K, Gel30K or Gel25K according to the  $M_n$  of  
4 CMCS.

## 5 6 2.5 Preparation of TP5 loaded hydrogels

7 TP5 loaded hydrogels were prepared through *in situ* encapsulation. In brief, 2.625 mg  
8 TP5 was dissolved in 0.5 mL CMCS80K (0.15 M). The resulting solution was mixed  
9 with 0.375 mL Dy solution (0.05 M). The glucosamine of CMCS to Dy molar ratio was  
10 4/1. After removal of bubbles, gelation then proceeded at 37 °C for 24 h to yield a TP5-  
11 loaded hydrogel with TP5 concentration of 3 mg/mL, namely Gel80K-TP5-3. Other  
12 TP5 loaded hydrogels were prepared using the same procedure but changing one  
13 parameter: Gel80K-TP5-0.1, Gel80K-TP5-9, Gel80K-TP5-3 (25°C) whose gelation  
14 occurred at 25°C, and Gel80K-TP5-3B prepared by dissolving TP5 in Dy solution  
15 before mixing with CMCS80K solution. TP5 was also loaded in Gel30K or Gel25K,  
16 and the corresponding samples were named as Gel30K-TP5-3 or Gel25K-TP5-3,  
17 respectively. **In situ incorporation of TP5 results in complete drug encapsulation.**  
18 **Therefore, the drug loading capacity of all the TP5-loaded hydrogels is 100 %.**

19

## 20 2.6 Characterization

21 <sup>1</sup>H NMR spectroscopy was carried out using Bruker NMR spectrometer (400-LS) at  
22 400 MHz. D<sub>2</sub>O was used as the solvent. 5 mg of sample were dissolved in 0.5 mL of  
23 solvent for each analysis.

24 Gel permeation chromatography (GPC) was performed on a Water 1515 multidetector  
25 GPC system equipped with Waters 410 differential detector and ultra-hydrogel 250  
26 column. 0.1 M NaCl was used as the mobile phase with a flow velocity of 0.6 mL/min.  
27 The column temperature was set at 40 °C, and the sample concentration was 5.0 mg/mL.  
28 Poly(ethylene glycol) standards were used for calibration.

1 Fourier-transform infrared (FT-IR) spectroscopy of freeze-dried hydrogels was  
2 conducted on Nicolet Nexus FT-IR spectrometer, equipped with ATR diamant Golden  
3 Gate.

4 The morphology of freeze-dried hydrogels was examined by using Hitachi S4800  
5 scanning electron microscopy (SEM). As-prepared hydrogels were placed in small  
6 vials, and immersed in liquid nitrogen (-196 °C) in order to conserve the original  
7 structure. The frozen samples were lyophilized with LABCONCO® freeze dryer for 24  
8 h, and sputter coated prior to analysis.

9 Physical MCR 301 Rheometer (Anton Paar) was utilized to determine the rheological  
10 properties of hydrogels, using a cone plate geometry (diameter of 4 cm, apex angle of  
11 2°, and clearance of 56 µm). The storage modulus ( $G'$ ) and loss modulus ( $G''$ ) were  
12 measured as a function of time, strain or frequency.

13

#### 14 2.7 Swelling of freeze-dried hydrogels

15 The swelling performance of freeze-dried gels was evaluated by immersion in buffer  
16 solutions at different pH values. Buffers from pH 3 to pH 7.4 were prepared from 0.1  
17 M citric acid and 0.2 M disodium hydrogen phosphate dodecahydrate solutions, buffers  
18 at pH 8 and pH 9 from 0.2 M boric acid and 0.05 M borax solutions, and buffer at pH  
19 10 from 0.05 M borax and 0.2 M sodium hydroxide solutions. Freeze-dried gel samples  
20 were immersed in a buffer solution, and taken out after 24 h. The swollen hydrogels  
21 were weighed after wiping surface water with filter paper, and weighed again after 24  
22 h lyophilization. The swelling and mass loss ratios of hydrogels were calculated  
23 according to the following equations:

$$24 \text{ Swelling ratio } \% = \frac{(M_s - M_d)}{M_d} \times 100 \quad (\text{Eq. 1})$$

$$25 \text{ Mass loss ratio } \% = \frac{(M_0 - M_d)}{M_0} \times 100 \quad (\text{Eq. 2})$$

26 Where  $M_0$  is the initial mass of dried gel,  $M_s$  is the wet mass of the swollen hydrogel,  
27 and  $M_d$  is the dried mass of the swollen hydrogel after lyophilization.

28

## 1 2.8 *In vitro* release of TP5

2 *In vitro* drug release was realized in pH = 7.4 phosphate buffered saline (PBS) at 37 °C.  
3 TP5-loaded hydrogel samples were placed in a centrifuge tube (50 mL) containing 10  
4 mL pH 7.4 PBS. *In vitro* release was performed in shaking water bath at 37 °C. At preset  
5 time intervals, 3 mL of the buffer solution were taken out, and replaced by 3 mL fresh  
6 buffer. TP5 concentration of the release supernatant was determined by HPLC-UV. A  
7 calibration curve was previously established using TP5 solutions with known  
8 concentrations. *In vitro* release of TP5 was also conducted in acidic buffer (pH 5.5),  
9 using the same procedure.

10 The cumulative release of TP5 was calculated using equation 3:

$$11 \text{ Release rate} = \frac{V_e \sum_{i=1}^{n-1} C_i + V_0 C_n}{M_{drug}} \times 100 \quad (\text{Eq. 3})$$

12 Where  $C_n$  and  $C_i$  represent the drug concentrations in the supernatant for  $n$  and  $i$   
13 withdrawing steps, respectively;  $V_0$  is initial total volume of PBS;  $V_e$  is collected  
14 volume of PBS;  $M_{drug}$  is the original loading amount of TP5. The drug loading  
15 efficiency is 100 % due to the *in situ* encapsulation of TP5. All of the experiments were  
16 performed in triplicate, and values were given in mean  $\pm$  SD for  $n = 3$ .

17

## 18 2.9 The release kinetics model

19 The release kinetics of the various TP5-loaded hydrogel systems were studied by fitting  
20 the *in vitro* release data using Higuchi's model [37, 38]:

$$21 Q_t = K_H \times t^{\frac{1}{2}} \quad (\text{Eq. 4})$$

22 where  $Q_t$  is the quantity of drug released within the time  $t$ ,  $K_H$  is the Higuchi dissolution  
23 constant.

24

## 25 2.10 Density functional theory (DFT) computation

26 All calculations were performed using the Dmol<sup>3</sup> code in the Materials Studio 7.0  
27 package [39]. The GGA/PBE functional was selected, and double numerical plus  
28 polarization (DNP) was used as the basis set [40]. Convergence tolerances for the total

1 energy, maximum force, and maximum displacement were set to  $1.0 \times 10^{-5}$  Hartree,  $2.0$   
2  $\times 10^{-3}$  Hartree/Å, and  $5.0 \times 10^{-3}$  Hartree/Å, respectively. The self-consistent field  
3 convergence was  $1.0 \times 10^{-6}$  Hartree. To accelerate the self-consistent field (SCF)  
4 convergence, the smearing was set to 0.005 Hartree. The solvent effect was simulated  
5 by conductor-like screening model (COSMO) [41], using the dielectric constant of  
6 78.54 (water). The transition states were calculated by using the synchronous method  
7 with conjugated gradient refinements. This method involves linear synchronous transit  
8 (LST) maximization, followed by repeated conjugated gradient (CG) minimizations,  
9 then quadratic synchronous transit (QST) maximizations and repeated CG  
10 minimizations until a transition state is located [42]. The energy barrier ( $E_{Barrier}$ ) was  
11 calculated using equation 5:

$$12 \quad E_{Barrier} = E_{TS} - E_{IN} \quad (\text{Eq. 5})$$

13 where  $E_{TS}$  is the energy of the transition state, and  $E_{IN}$  the energy of the intermediate  
14 connected to the transition state.

15 The reaction energy ( $E_{RE}$ ) of the elementary steps was calculated using equation 6:

$$16 \quad E_{RE} = E_P - E_R \quad (\text{Eq. 6})$$

17 where  $E_P$  is the energy of the elementary reaction product, and  $E_R$  the initial energy  
18 of the elementary reaction reactant.

19

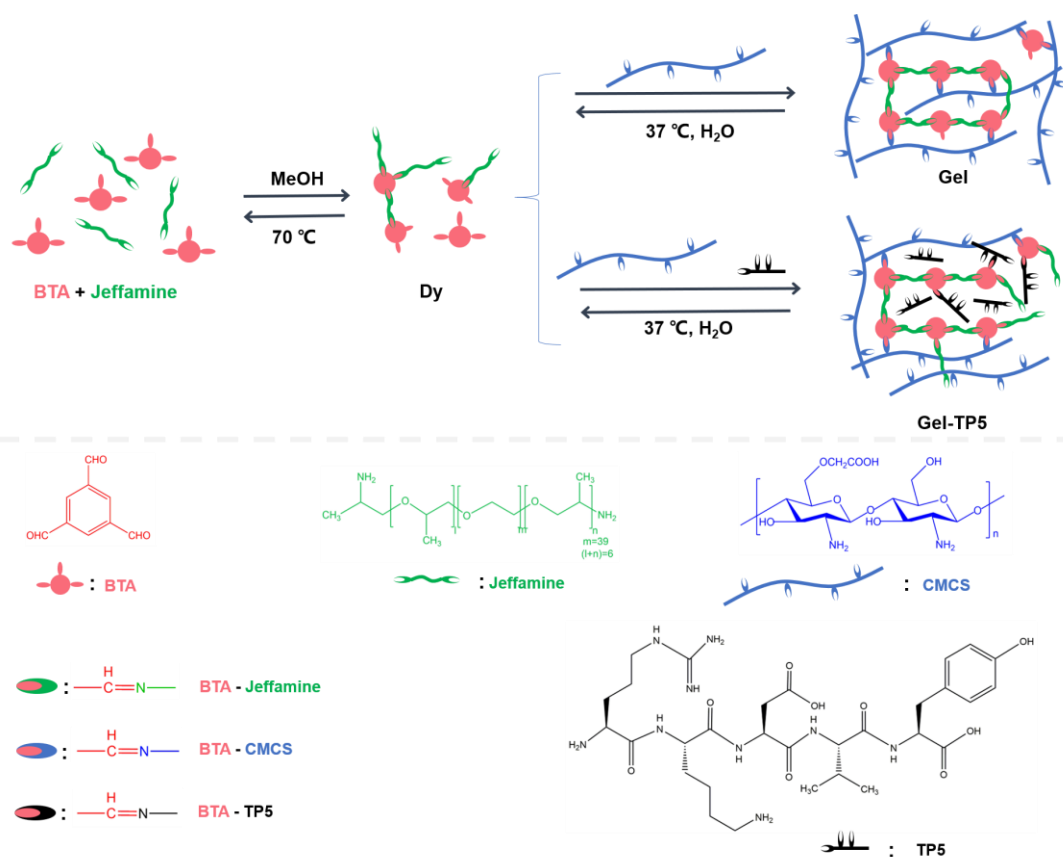
### 20 **3. Results and discussion**

#### 21 **3.1 Preparation of hydrogels**

22 Dynamic hydrogels were synthesized via Schiff-base reaction between CMCS and the  
23 dynamer, as shown in Scheme 1. Upon mixing CMCS and Dy aqueous solutions, the  
24 amino groups along the CMCS backbone react with the remaining aldehyde groups of  
25 Dy, resulting in formation of a 3D hydrogel network by dual imine bonding. The CMCS  
26 to Dy molar ratio of 4:1 was adopted since it allows optimal crosslinking, as  
27 demonstrated in our previous work [33]. In situ TP5 encapsulation in hydrogels is also  
28 shown in Scheme 1. Table 1 summarizes all the details for the preparation of blank and

1 drug loaded hydrogels.

2



3

4 **Scheme 1.** Synthesis of dynamic hydrogels via Schiff-base reaction between CMCS

5 and Dy, and in-situ loading of TP5 in hydrogels.

6

7 **Table 1.** Molar and mass compositions of blank and TP5 loaded hydrogels <sup>a)</sup>

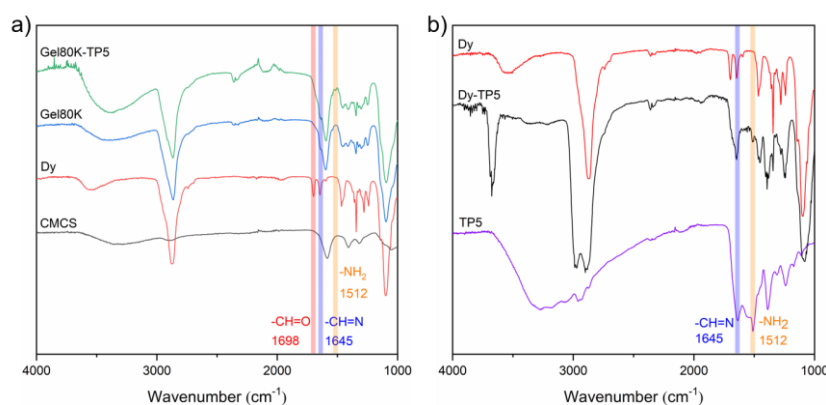
Sample	CMCS80K		CMCS30K		CMCS25K		Dy <sup>c)</sup>		TP5 <sup>d)</sup>
	[mmol]	[w/v%]	[mmol]	[w/v%]	[mmol]	[w/v%]	[mmol]	[w/v%]	
	b)		b)		b)				
Gel80K	0.3	1.8	-	-	-	-	0.075	4.3	-
Gel30K	-	-	0.3	1.8	-	-	0.075	4.3	-
Gel25K	-	-	-	-	0.3	1.8	0.075	4.3	-
Gel80K-TP5- 0.1	0.3	1.8	-	-	-	-	0.075	4.3	0.1



Gel80K-TP5-3	0.3	1.8	-	-	-	-	0.075	4.3	3
Gel80K-TP5-3B <sup>d)</sup>	0.3	1.8	-	-	-	-	0.075	4.3	3
Gel80K-TP5-3 (25 °C) <sup>e)</sup>	0.3	1.8	-	-	-	-	0.075	4.3	3
Gel80K-TP5-9	0.3	1.8	-	-	-	-	0.075	4.3	9
Gel30K-TP5-3	-	-	0.3	1.8	-	-	0.075	4.3	3
Gel25K-TP5-3	-	-	-	-	0.3	1.8-	0.075	4.3	3

1 a) Hydrogels were prepared by mixing the aqueous solutions of 0.15 M CMCS80K/CMCS30K/CMCS25K and 0.05  
2 M Dy at a D-glucosamine/Dy molar ratio of 4/1 (total volume of 3.5 mL), TP5 loaded hydrogels were prepared by  
3 adding TP5 in CMCS solution before mixing with Dy solution; b) Moles of glucosamine units of CMCS.  
4 Calculations were made on the basis of the average molar mass of 212 g/mol obtained for D-glucosamine, taking  
5 into account the degree of deacetylation of 90% and the degree of carboxymethylation of 80%; c) The concentration  
6 of Dy solution was obtained from the remaining aldehyde groups. In a typical reaction, 0.5 mmol BTA (81 mg)  
7 reacted with 0.5 mmol Jeffamine ED2003 (950 mg) to form a Dy solution. As BTA had 3 aldehydes and Jeffamine  
8 2 amines, there remained theoretically 0.5 mmol of aldehydes in the dried Dy. Addition of 10 mL water yielded a  
9 dynamer solution of 0.05 M. d) Gel80K-TP5-3B was prepared by adding TP5 in Dy solution before mixing with  
10 CMCS solution e) Gel80K-TP5-3 (25 °C) was prepared at 25 °C; f) The concentration of TP5 in hydrogel was 0.1  
11 mg/ml or 3 mg/ml or 9 mg/ml.

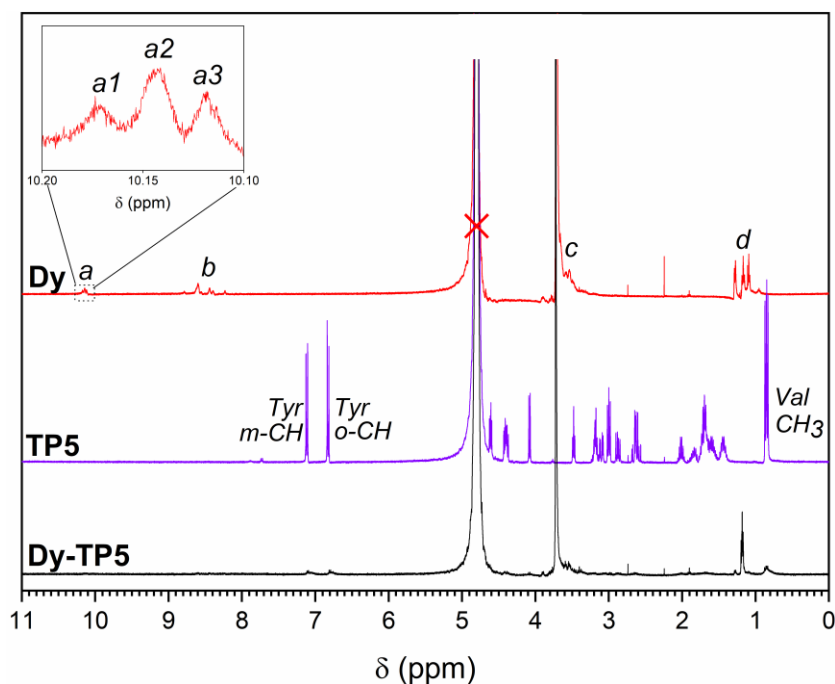
### 13 3.2 FT-IR and NMR



14  
15 **Fig. 1.** a) FT-IR spectra of freeze-dried CMCS, Dy, Gel80K and Gel80K-TP5-3; b) FT-  
16 IR spectra of Dy, TP5, and Dy-TP5 which is an obtained by mixing TP5 in Dy solution  
17 at a molar ratio of 1/1, reaction at 37°C for 24h, and 24 h lyophilization.

18 Fig. 1a shows the FTIR spectra of CMCS, Dy, freeze dried Gel80K and TP5 loaded

1 Gel80K-TP5-3. CMCS presents a large band around  $3350\text{ cm}^{-1}$  which is attributed to  
2 free OH and  $\text{NH}_2$  groups, and an intense band at  $1595\text{ cm}^{-1}$  due to carbonyl groups. The  
3 dynamer exhibits two intense bands at  $2850$  and  $1100\text{ cm}^{-1}$  assigned to C-H and C-O  
4 stretching, and two characteristic bands at  $1698$  and  $1645\text{ cm}^{-1}$  corresponding to  
5 aldehyde and imine bonds, respectively. All the characteristic bands of CMCS and Dy  
6 are observed on the spectrum of freeze dried Gel80K. Nevertheless, the band of  
7 aldehyde groups at  $1698\text{ cm}^{-1}$  is almost invisible, but the band at  $1645\text{ cm}^{-1}$   
8 corresponding to newly formed imine bonds is clearly distinguished. These results  
9 illustrate that hydrogels are formed due to imine formation between aldehydes of Dy  
10 and amine groups of CMCS. On the spectrum of Gel80K-TP5-3, a band is detected at  
11  $2100\text{ cm}^{-1}$ , which is assigned to the O-H stretching of water [43]. In fact, the freeze-  
12 dried samples rapidly absorbed water due to the high hygroscopicity of TP5 [44, 45].  
13 Similar findings are obtained for other blank and TP5 loaded hydrogels (Fig. S1).  
14 Fig. 1b presents the FT-IR spectra of Dy, TP5 and a Dy-TP5 mixture. The latter was  
15 prepared in order to figure out the reactions between the aldehydes of Dy and the amines  
16 of TP5. It was obtained by dissolving TP5 in Dy solution at a molar ratio of 1/1,  
17 followed by 24 h reaction at  $37\text{ }^\circ\text{C}$  and finally 24 h lyophilization. TP5 presents  
18 characteristic bands at  $1630$  and  $1512\text{ cm}^{-1}$  which are assigned to the C=O stretching of  
19 amides and N-H pending of amines, together with a large band around  $3300\text{ cm}^{-1}$  due  
20 to the presence of water. Interestingly, the band of aldehydes at  $1698\text{ cm}^{-1}$  is not detected  
21 on the spectrum of Dy-TP5, suggesting that the aldehydes have reacted with the amine  
22 groups of TP5 via Schiff-base reaction.  
23



1  
2 **Fig. 2.**  $^1\text{H}$  NMR spectra of the Dy, TP5, and Dy-TP5 in  $\text{D}_2\text{O}$ .

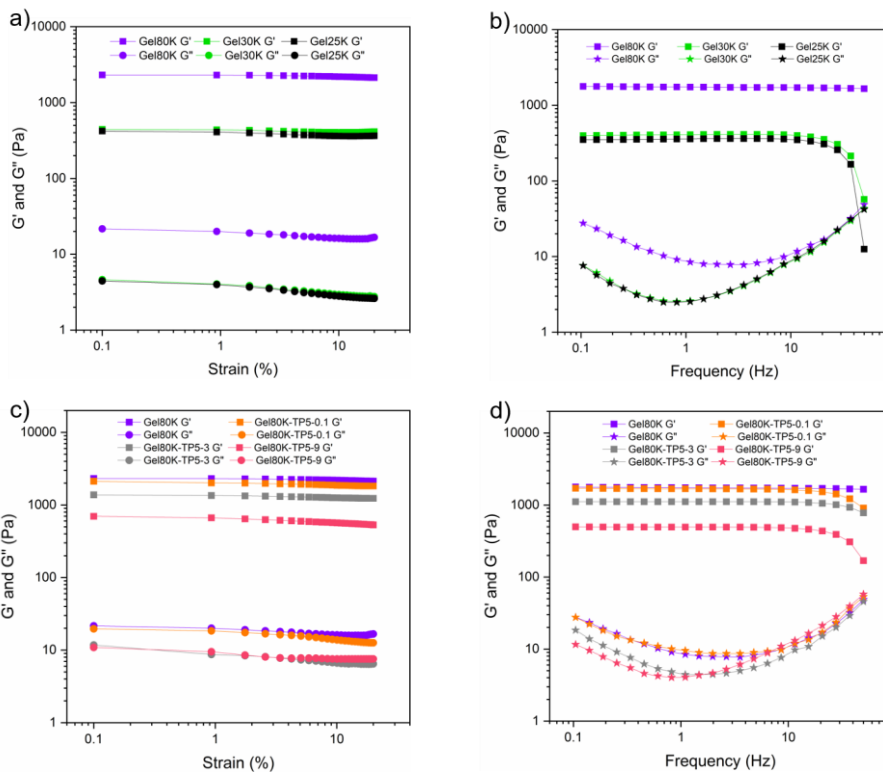
3  
4 Fig. 2 shows the  $^1\text{H}$  NMR spectra of the Dy, TP5, and Dy-TP5. On the spectrum of Dy,  
5 the three small signals in the 10.1-10.2 ppm range are assigned to aldehyde groups of  
6 BTA with different degrees of substitution, including non-substituted (a1), mono-  
7 substituted (a2), and di-substituted aldehydes (a3). Signals between 8.2 and 8.7 ppm  
8 belong to the aromatic protons of BTA and imine protons (b). Signals around 3.7 ppm  
9 are assigned to the methylene and methine protons (c) of Jeffamine, and signals between  
10 1.0 and 1.3 ppm to the methyl protons (d) of Jeffamine [33, 34]. The presence of  
11 residual  $\text{H}_2\text{O}$  is detected at 4.8 ppm. TP5 presents numerous signals associated to the  
12 various protons of the pentapeptide (Arg-Lys-Asp-Val-Tyr). The two doublets around  
13 7.15 and 6.85 ppm are assigned to the meta-CH (Tyr m-CH) and ortho-CH (Tyr o-CH)  
14 on the phenyl ring of tyrosine [46], respectively. The three signals between 4.0 and 4.7  
15 ppm belong to the various methine protons, and the signals between 1.3 and 3.6 ppm to  
16 the various methylene protons of the pentapeptide. The strong signal at 0.85 ppm is  
17 attributed to the methyl protons of valine (Val  $\text{CH}_3$ ) [46]. Importantly, no signals in the  
18 range of 10.1-10.2 ppm are observed on the spectrum of Dy-TP5, implying that all the  
19 remaining aldehydes of Dy have completely reacted with the amine groups of TP5, in

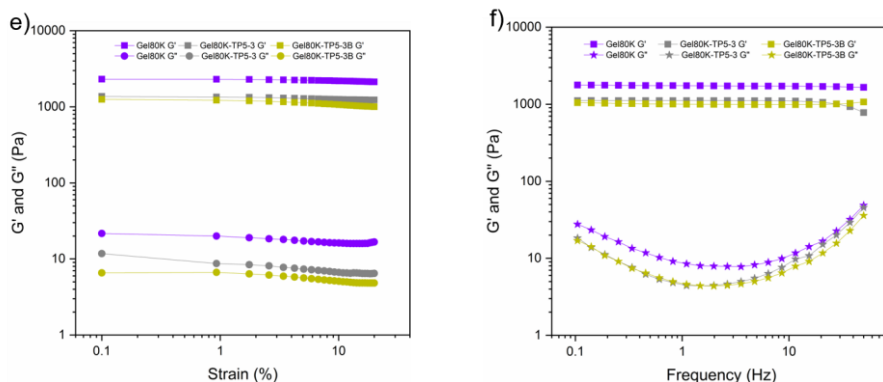
1 agreement with FTIR data (Fig. 1b).

2 The morphology of CMCS, Dy, their mixtures with TP5 (CMCS-TP5 and Dy-TP5) was  
3 examined by using SEM (Fig. S2). No obvious difference is observed between pristine  
4 CMCS (Fig. S2a, b) and CMCS-TP5 (Fig. S2e, f). Both samples display a lamellar  
5 structure with the presence of holes of *c.a* 10  $\mu\text{m}$ , suggesting the absence of interactions  
6 between the two components. In contrast, difference is detected between pristine Dy  
7 (Fig. S2c, d) and the mixture Dy-TP5 (Fig. S2g, h). Both samples exhibit a highly  
8 porous structure. The surface of Dy appears rugged, whereas that of Dy-TP5 appears  
9 smooth. These findings seem to indicate the occurrence of interactions between TP5  
10 and the Dy.

11

### 12 3.3 Rheology





1  
2 **Fig. 3.** Storage modulus ( $G'$ ) and loss modulus ( $G''$ ) changes of Gel80K, Gel30K and  
3 Gel25K as a function of strain at 25 °C and at 1 Hz (a) and as a function of frequency  
4 at 25 °C and at a strain of 1 % (b);  $G'$  and  $G''$  changes of Gel80K, , Gel80K-TP5-0.1,  
5 Gel80K-TP5-3 and Gel80K-TP5-9 as a function of strain at 25 °C and at 1 Hz (c) and  
6 as a function of frequency at 25 °C and at a strain of 1% (d);  $G'$  and  $G''$  changes of  
7 Gel80K, Gel80K-TP5-3, and Gel80K-TP5-3B as a function of strain at 25 °C and at 1  
8 Hz (e) and as a function of frequency at 25 °C and at a strain of 1 % (f).

9  
10 Rheological measurements were conducted on the various hydrogels at 25 °C to  
11 evidence the effect of CMCS molar mass on the rheological properties as well as the  
12 interaction between TP5 and Dy in the hydrogel network. Firstly, the storage modulus  
13 ( $G'$ ) and loss modulus ( $G''$ ) changes of blank hydrogels (Gel80K, Gel30K and Gel25K)  
14 were determined as a function of strain at a frequency of 1 Hz and at 25 °C, as shown  
15 in Fig. 3a. As the strain increases from 0.1 % to 20 %,  $G'$  remains almost constant for  
16 all the blank hydrogels, whereas  $G''$  slightly decreases, indicating a linear viscoelastic  
17 (LVE) behavior of hydrogels in the 0.1 to 20 % strain range. Meanwhile, the moduli of  
18 blank hydrogels are highly dependent on the molar mass of CMCS. With the decrease  
19 of molar mass of CMCS from 80,000 to 30,000 and to 25,000,  $G'$  at a strain of 1%  
20 decreases from 2300 Pa for Gel80K to 440 Pa for Gel30K, and to 420 Pa for Gel25K,  
21 respectively. Similar phenomena are observed for  $G''$  changes. In fact, higher molar  
22 mass chains have more amino groups, which allows to enhance the crosslinking density,  
23 thus resulting in higher modulus of hydrogels

1 Fig. 3b shows the modulus changes of blank hydrogels as a function of frequency from  
2 0.1 Hz to 50 Hz at a constant strain (1%) and at 25 °C.  $G'$  remains constant in the whole  
3 frequency range for Gel80K, whereas a sharp decrease of  $G'$  is observed beyond 30 Hz  
4 in the cases of Gel30K and Gel25K. On the other hand,  $G''$  exhibits some fluctuations  
5 with increasing frequency. These rheological results well corroborate with the  
6 formation of highly stable covalent networks, in contrast to physical hydrogels whose  
7 storage and loss moduli increase with increasing frequency due to chain entanglements  
8 [19, 47]. It is also noteworthy that Gel80K exhibit higher stability than Gel30K and  
9 Gel25K due to higher crosslinking density as mentioned above.

10 Fig. 3c presents  $G'$  and  $G''$  changes *versus* strain of Gel80K hydrogels containing  
11 various amounts of TP5 (0, 0.1, 3, and 9 mg/mL). Obviously, with addition of TP5 in  
12 hydrogels,  $G'$  decreases from 2310 Pa for Gel80K to 2222 Pa for Gel80K-TP5-0.1, to  
13 1374 Pa for Gel80K-TP5-3, and to 697 Pa for Gel80K-TP5-9. These results illustrate  
14 that *in situ* TP5 encapsulation affects the rheological properties of drug loaded  
15 hydrogels. In fact, the amine groups of TP5 can react with the free aldehyde groups of  
16 Dy, as shown in Fig. 1b and Fig. 2. Hence, there is competition between TP5 and CMCS  
17 after mixing aqueous solution of Dy with that of CMCS and TP5, leading to decreased  
18 imine bonding between CMCS and Dy. On the other hand, attachment of TP5 to the  
19 network does not contribute to the crosslinking because TP5 is a small molecule  
20 compared to CMCS. Therefore, the crosslinking density decreases with increase of TP5  
21 content, and consequently the modulus of hydrogels decreases. A frequency sweep of  
22 hydrogels was performed over a range from 0.01 to 50 Hz at a fixed strain of 1 %.  $G'$   
23 remains constant for Gel80K, and slightly decreases beyond 30 Hz for TP5 loaded  
24 hydrogels, indicating that TP5 encapsulation affects the stability of hydrogels.

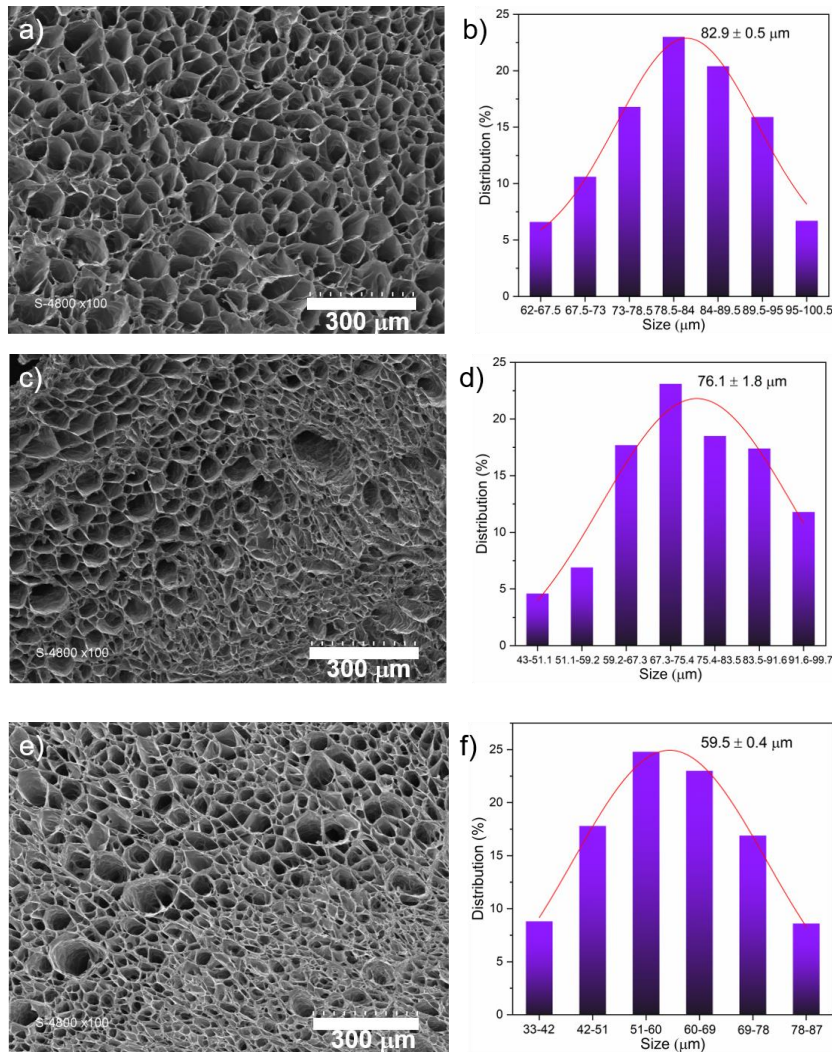
25 A hydrogel (Gel80K-TP5-3B) was prepared by dissolving TP5 in Dy solution,  
26 followed by mixing with CMCS solution before gelation. As TP5 is supposed to react  
27 with Dy, dissolving TP5 in Dy solution should favor imine formation between them.  $G'$   
28 and  $G''$  changes of Gel80K, Gel80K-TP5-3, and Gel80K-TP5-3B were examined as a

1 function of strain and frequency (Fig. 3e, f). Noticeably,  $G'$  of Gel80K-TP5-3 is higher  
 2 than that of Gel80K-TP5-3B in nearly the whole strain and frequency ranges, which  
 3 confirms that mixing TP5 and Dy first decreased the crosslinking density of hydrogels.  
 4 Strain and frequency sweeps were also performed on blank and TP5 loaded Gel30K  
 5 and Gel25K hydrogels (Fig. S3). Similar phenomena were observed, illustrating the  
 6 stability of hydrogels and interactions between TP5 and the dynamer.

7

### 8 3.4 Morphology and swelling properties

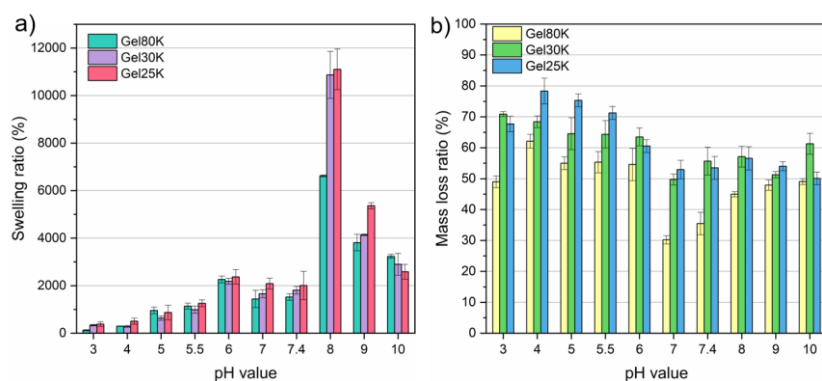
9



13 **Fig. 4.** SEM images and pore size distributions of freeze-dried gels: (a, b) Gel80K; (c,  
 14 d) Gel30K; (e, f) Gel25K.



1 The morphology of as-prepared hydrogels after lyophilization was observed by SEM.  
2 Obviously, all hydrogels exhibit interpenetrating and porous architectures. Compared  
3 to Gel30K and Gel25K, Gel80K displays more regular structure and narrower  
4 distribution of interconnected pores (Fig. 4a). The average pore diameter of Gel80K,  
5 Gel30K and Gel25K is  $82.9 \pm 0.5 \mu\text{m}$  (Fig. 4b),  $76.1 \pm 1.8 \mu\text{m}$  (Fig. 4d), and  $59.5 \pm 0.4$   
6  $\mu\text{m}$  (Fig. 4f), respectively. Thus, it seems that the pore diameter slightly decreases with  
7 decrease of the molar mass of CMCS. Moreover, in a previous work, it was shown that  
8 the pore diameter of dynamic hydrogels is dependent on the molar mass of Jeffamine  
9 linker [34]. Therefore, the porous structure of dynamic hydrogels can be tailored by  
10 varying the molar mass of the components. On the other hand, the interconnected  
11 porous structure enables hydrogels to mimic the extracellular matrix, which is  
12 beneficial for biomedical applications in drug delivery [48], and in tissue engineering  
13 [33, 49]. Therefore, Gel80K which has the optimal porous “spongy” characteristics was  
14 selected as the carrier of an in-depth study of drug release.  
15



16  
17 **Fig. 5.** a) Swelling ratios, and b) Mass loss ratios of Gel80K, Gel30K and Gel25K after  
18 24 h immersion in buffers at various pH values. Data are expressed as mean  $\pm$  SD for n  
19 = 3.

20 As aforementioned, the most important characteristic of hydrogels is their capability to  
21 retain large amount of water in the polymeric network, which is beneficial for  
22 controlling the release profiles of drugs and the absorption of wound exudate [50]. Fig.  
23 5a presents the swelling performance of freeze-dried hydrogels in buffers at various pH



1 values. All the hydrogels exhibit a highly pH-dependent swelling behavior. The  
2 swelling degree is relatively low ( $< 1000\%$ ) in strong acid buffers (pH 3/4), and  
3 increases to around  $2000\%$  from pH 4 to 6. It remains at the same level around  $2000\%$   
4 in the pH 6 to 7.4 range. All hydrogels exhibit a maximum swelling at pH 8,  $6600\%$   
5 for Gel80K,  $10000\%$  for Gel30K, and  $11000\%$  for Gel25K. As the alkalinity continues  
6 to increase to pH 9 and 10, the swelling degree of hydrogel decreases dramatically to a  
7 level around  $3000\%$ . In addition, there is no major difference between the swelling  
8 ratios of the three samples except at pH 8. The pH sensitivity of the swelling of  
9 hydrogels is assigned to electrostatic interactions between the amino and carboxyl  
10 groups along CMCS chains [33, 51]. In fact, the  $pK_a$  of amino and carboxyl groups of  
11 CMCS is 6.5 and 2.7, respectively. Contraction of hydrogels occurs because of strong  
12 electrostatic attraction between negatively charged  $-\text{COO}^-$  and positively charged  $-\text{NH}_3^+$   
13 groups which result from protonation in strong acidic media (pH 3 and 4), thus leading  
14 to low swelling ratio. Electrostatic attraction diminishes with the decrease of the  $\text{H}^+$   
15 concentration (pH 5, 5.5, and 6), and thus the swelling gradually increases. At pH 6, 7  
16 and 7.4 which are around the  $pK_a$  of amino groups, the swelling remains almost constant  
17 as the electrostatic interactions are weak due to low concentrations of charges. In  
18 contrast, high swelling of hydrogels occurs because of strong repulsion between  
19 negatively charged  $-\text{COO}^-$  groups at pH 8. Nevertheless, with higher alkalinity at pH 9  
20 and 10,  $\text{OH}^-$  ions in solution would counterbalance the electrostatic repulsion between  
21  $-\text{COO}^-$  groups [33], thus resulting in decrease of the swelling degree. The pH sensitive  
22 swelling of hydrogels could be of great importance for the design of drug delivery  
23 systems due to large variation of physiological pH at various body sites in normal and  
24 pathological conditions [52].

25 Mass loss of hydrogels occurs during the swelling process due to the solubilization and  
26 washing away of non-crosslinked species, as shown in Fig. 5b. Overall, the mass loss  
27 ratio of all the hydrogels varies between  $30\%$  and  $80\%$  in the whole pH range. The  
28 hydrogels show higher mass loss in acid conditions than in neutral and alkaline

1 conditions. Noticeably, the mass loss ratio of Gel80K is lower than those of Gel30K  
2 and Gel25K in the whole pH range. This finding could be attributed to the better cross-  
3 linking Gel80K as compared to Gel30K and Gel25K because Gel80K was prepared  
4 from CMCS80K with higher molar mass, in agreement with rheological data. The  
5 lowest mass loss ratio is 30 and 36 % obtained for Gel80K at pH 7 and 7.4, respectively,  
6 in agreement with the better stability of Gel80K at neutral pH. Thus, Gel80K is used  
7 for a comprehensive study of TP5 release from hydrogels under various conditions.  
8 The swelling and mass loss behaviors of Gel80K were investigated under drug release  
9 conditions in pH 7.4 PBS at 37 °C up to 168 h (Fig. S4, Information supplementary).  
10 The swelling ratio rapidly increases to 750 % and 1200 % after immersion for 10 and  
11 20 min, respectively, whereas the mass loss ratio increases 25 % and 32 % in the  
12 meantime. Thereafter, the swelling and mass loss ratios remain almost at the same level  
13 up to 168 h, fluctuating around 1300 % and 34 %, respectively. These findings indicate  
14 that an equilibrium is rapidly reached after immersion of dried gels in PBS, and that the  
15 hydrogels are stable after initial mass loss.

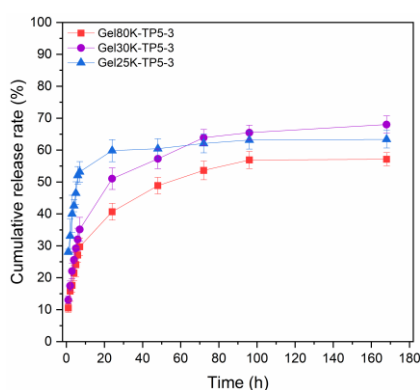
16

### 17 **3.5 *In vitro* release of the TP5**

18 TP5 is an immuno-modulating drug, and is widely applied in the treatment of  
19 autoimmune diseases such as chronic lymphocytic leukemia [10], Sezary's syndrome  
20 [53, 54], etc. Besides, TP-5 is able to induce the differentiation of T-cells and accelerate  
21 the development of T lymphocytes [11]. A series of TP5-loaded hydrogels were  
22 prepared, including Gel80K-TP5-0.1, Gel80K-TP5-3, Gel80K-TP5-9, Gel80K-TP5-  
23 3B, Gel80K-TP5-3(25°C), Gel30K-TP5-3, and Gel25K-TP5-3 so as to evaluate the  
24 effects of various factors.

25 The morphology of the various TP5-loaded hydrogels was examined by using SEM  
26 (Fig. S5). It appears that TP5 encapsulation affects the regularity of the porous structure  
27 of hydrogels. In fact, Gel80K-TP5-0.1 exhibits a porous structure similar to that of  
28 Gel80K (Fig. 4a,b), but the surface appears rougher. With increase of TP5 content from

1 0.1 mg/mL (Fig. S5a, b) to 3 mg/mL (Fig. S5c, d), and to 9 mg/mL (Fig. S5e, f), the  
2 three-dimensional architecture becomes more and more irregular as compared with that  
3 of Gel80K (Fig. 4a,b). Similarly, TP5 encapsulation also affects the structure of  
4 Gel30K-TP5-3 (Fig. S5i, j) and Gel25K-TP5-3 (Fig. S5k, l) as compared to  
5 corresponding blank hydrogels. On the other hand, difference is also noticed between  
6 the morphology of Gel80K-TP5-3 and Gel80K-TP5-3B (Fig. S5g, h). Both have  
7 exactly the same compositions, but different mixing orders. Gel80K-TP5-3B exhibits a  
8 smoother surface compared to Gel80K-TP5-3.

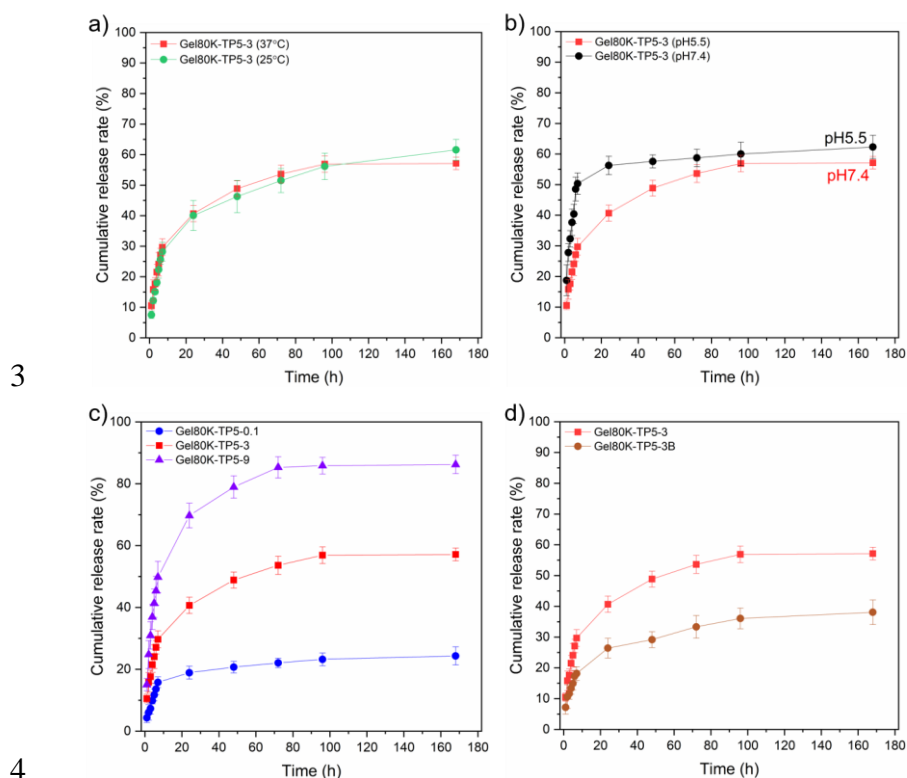


9  
10 **Fig. 6.** Cumulative release of TP5 from Gel80K-TP5-3, Gel30K-TP5-3, and Gel25K-  
11 TP5-3. Values were expressed as mean  $\pm$  SD for  $n = 3$ .

12

13 TP5 release from the various TP5-loaded hydrogels was monitored to evaluate the  
14 potential of dynamic hydrogels as drug carrier in cancer therapy [38]. Fig. 6 shows the  
15 release profiles of TP5 from Gel80K-TP5-3, Gel30K-TP5-3, and Gel25K-TP5-3 in pH  
16 7.4 PBS at 37 °C. Overall, all samples displayed a release profile in two stages, namely  
17 a burst release in the first 7 h followed by a slow and continuous release during 7 days.  
18 After 1 h in the medium, significant release rates are recorded, 28.0% for Gel25K-TP5,  
19 13.0 % for Gel30K-TP5 and 10.5 % for Gel80K-TP5. TP5 release increases almost  
20 linearly to reach 53.2 %, 35.1 % and 29.7 % after 7 h for the 3 samples, respectively.  
21 Thereafter, TP5 release slows down, reaching a plateau after 96 h. The total TP5 release  
22 is 63.4, 68.0, and 57.1% after 168 h for Gel25K-TP5, Gel30K-TP5 and Gel80K-TP5,  
23 respectively. Gel80K-TP5 exhibits slower and more sustainable release as compared to

1 Gel25K-TP5 and Gel30K-TP5 due to its higher crosslinking density which disfavors  
2 drug diffusion. Therefore, Gel80K is used as drug carrier in further studies.



6 **Fig. 7.** a) Cumulative release of TP5 from Gel80K-TP5-3 prepared at 25 °C and 37 °C,  
7 namely Gel80K-TP5-3 (25 °C) and Gel80K-TP5-3 (37 °C); b) Cumulative release of  
8 TP5 from Gel80K-TP5-3 in PBS at pH 7.4, and in acid buffer medium at pH 5.5, namely  
9 Gel80K-TP5-3 (pH 7.4) and Gel80K-TP5-3 (pH 5.5); c) Cumulative release of TP5  
10 from Gel80K loaded with various TP5 contents: Gel80K-TP5-0.1, Gel80K-TP5-3, and  
11 Gel80K-TP5-9; d) Cumulative release of TP5 from Gel80K-TP5-3 and Gel80K-TP5-  
12 3B with different mixing orders. Values were expressed as mean  $\pm$  SD for n = 3.

13  
14 Fig. 7 show the TP5 release curves from various hydrogels. No difference is noticed  
15 between the release profiles Gel80K-TP5-3 prepared at 25 °C and at 37 °C (Fig. 7a),  
16 indicating the gelation temperature (25 or 37 °C) of Gel80K-TP5 has little impact on  
17 the release of TP5. In contrast, faster initial release is observed from Gel80K-TP5-3 at  
18 pH 5.5 than at pH 7.4, although both present similar equilibrium release ratios around

1 60% (Fig. 7b). This difference can be assigned to the fact that the imine bonds are prone  
 2 to hydrolysis under acidic condition [55], which leads to partial disassociation of the  
 3 three-dimensional hydrogel network thus favoring the diffusion of trapped TP5. It is  
 4 also of interest to evaluate the effect of initial drug load on the release rate by comparing  
 5 the release profiles of Gel80K-TP5-0.1, Gel80K-TP5-3, Gel80K-TP5-9, as shown in  
 6 Fig. 7c. Apparently, the latter shows the highest equilibrium rate, reaching 90 % after  
 7 168 h, which is much higher than that of Gel80K-TP5-0.1 (22.5%) and Gel80K-TP5-3  
 8 (57 %). This difference well corroborates with the partial attachment of TP5 to the  
 9 hydrogel network by imine bonding. It can be assumed that only free TP5 is released  
 10 as imine bonds are stable at pH 7.4. Thus, hydrogels with higher drug loading have  
 11 higher proportion of free drug, and consequently present higher drug release ratio [38].  
 12 Finally, TP5 release behaviors from Gel80K-TP5-3 and Gel80K-TP5-3B are compared  
 13 to examine the effect of the mixing order (Fig. 7d). Gel80K-TP5-3B presents a total  
 14 release ratio of 38%, which is much lower than that of Gel80K-TP5-3 (60%). This  
 15 finding also confirms the covalent attachment of TP5 to the hydrogel network by imine  
 16 bonding. As TP5 is first mixed with Dy before mixing with CMCS in the case of  
 17 Gel80K-TP5-3B, more TP5 is covalently attached to the network, leading to lower TP5  
 18 release as compared to Gel80K-TP5-3. These data indicate that only physically  
 19 encapsulated TP5 can be released under in vitro conditions. The release rate of TP5  
 20 from hydrogels depends on many factors such as the molar mass of CMCS, the pH of  
 21 the medium, the drug load, and the mixing order during hydrogel preparation.

22

### 23 3.6 Drug release kinetics

24 Table 2 Parameters obtained from fitting of experimental data using Higuchi model <sup>a)</sup>

Sample	R <sup>2</sup>	K <sub>H</sub>
Gel80K-TP5-0.1	0.967	6.477
Gel80K-TP5-3	0.990	11.110
Gel80K-TP5-9	0.998	20.897

Gel80K-TP5-3B	0.978	6.502
Gel80K-TP5 (pH5.5)	0.982	19.141
Gel80K-TP5 (25°C)	0.985	12.271
Gel30K-TP5	0.997	13.315
Gel25K-TP5	0.986	16.172

1 a) Experimental data in Fig. 6-7.

2

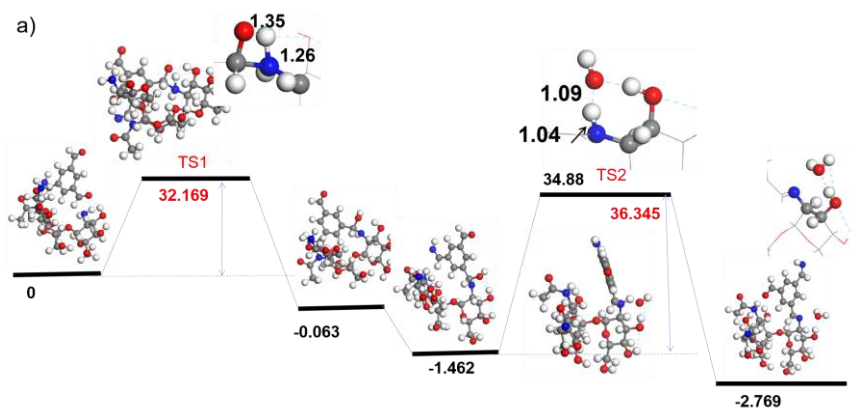
3 It is widely admitted that the release of a hydrophilic drug from a monolithic matrix  
 4 mainly depends on the diffusion process. Other factors such as physical forces between  
 5 the drug and the hydrogel matrix and swelling of bulk hydrogel affect the release profile  
 6 as well. Higuchi's model has been largely used to describe the drug release kinetics of  
 7 various systems. It is based on the Fick's Law where the release proceeds by the  
 8 diffusion of drugs through the delivery system. In this case, the cumulative released  
 9 drug amount is proportional to the square root of time. TP5 release kinetics were  
 10 investigated by fitting of experimental data in the first 6 h using Higuchi model in order  
 11 to better understand the release mechanism of TP5 from dynamic hydrogels [38], as  
 12 shown in Fig. S6 and Table 2.

13 Data in Table 2 show that the correlation coefficient is above 0.98 in all cases except  
 14 Gel80K-TP5-0.1, suggesting that the TP5 release was mainly controlled by diffusion  
 15 process [56]. In fact, the maximum concentration of TP5 after total release in the release  
 16 medium is well below its saturation concentration ( $> 10$  mg/mL) even for Gel80K-  
 17 TP5-9 [19]. Thus, it could be considered that the dissolution is the ruling force of TP5  
 18 release from the hydrogels as drug release was conducted under immersion conditions  
 19 [38]. It is also of interest to compare the  $K_H$  values of the different systems. Gel80K-  
 20 TP5-9 and Gel80K-TP5-0.1 present the highest and the lowest  $K_H$  values, respectively,  
 21 in agreement with the fastest and the slowest release rates. Intermediate  $K_H$  values were  
 22 obtained for other TP5 loaded hydrogels.

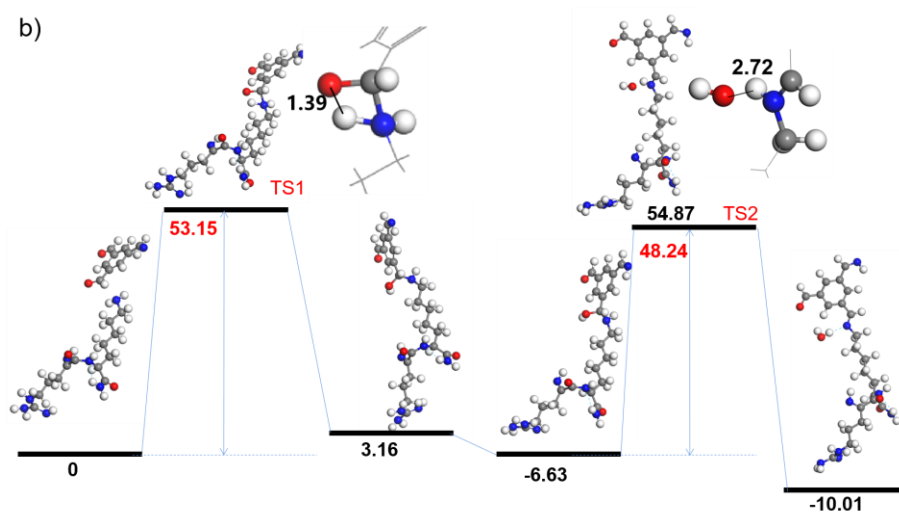
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### 24 **3.7 Density functional theory (DFT) modelling**

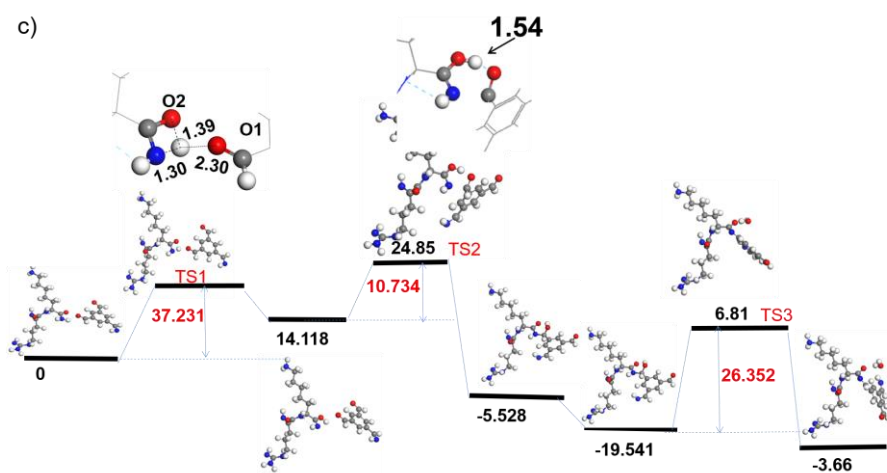
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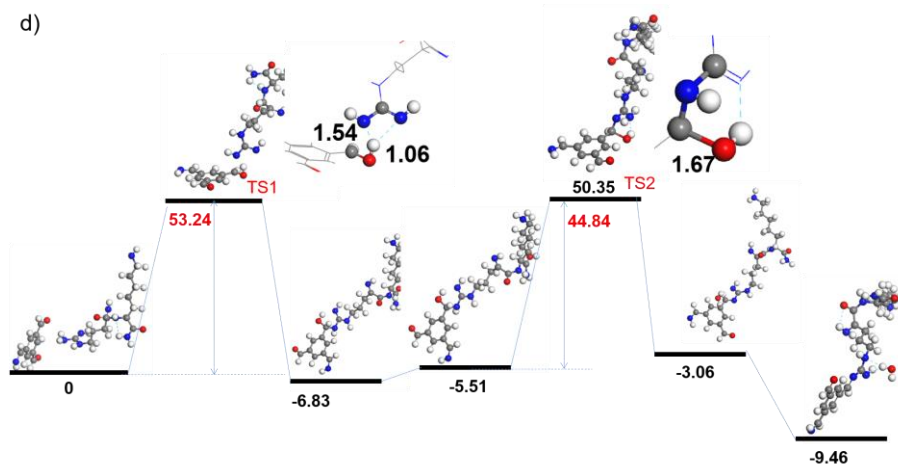
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3



4



**Fig. 8.** Computation by density functional theory (DFT) to determine the lowest reaction pathways between : a) CMCS-l-Dy; b) TP5(1)-l-Dy (TP5 site 1); c) TP5(2)-l-Dy (TP5 site 2); and d) TP5(3)-l-Dy (TP5 site 3). The reaction energy and energy barrier are calculated by Eq. 5 and Eq. 6 in kcal/mol.

Density functional theory (DFT) was used to determine the reaction energy and energy barrier of imine linkages between TP5 and Dy (TP5-l-Dy, ‘l’ stands for linkage, similarly hereinafter) as well as those between CMCS and Dy (CMCS-l-Dy. The simplified models of CMCS, Dy and TP5 are shown in Fig. S7a-c. Only one reaction site is considered taking into account the repeatability of the amino groups in CMCS and the symmetry of Dy. The lowest energy pathway of the Schiff-base reaction between CMCS and Dy is shown in Fig. 8a. The first step of the reaction is the dehydrogenation from the amino group of Dy, which requires overcoming an energy barrier of 32.2 kcal/mol. The formation of CH-NH bond is thermodynamically favorable. This is followed by the cleavage of the oxhydrile group, which needs higher energy than the first step, reaching 36.3 kcal/mol. The formation and removing of H<sub>2</sub>O molecule are thermodynamically favorable. Thus, the rate-limiting step of the reaction between CMCS and Dy is the cleavage of oxhydrile with an energy barrier of 36.3 kcal/mol.

There are three kinds of amino groups in TP5 which have different chemical environments (Fig. S7c). Thus, there are three -NH<sub>2</sub> sites that can be involved in the



1 Schiff-base reaction, which are marked as TP5(1)-l-Dy (Fig. 8b), TP5(2)-l-Dy (Fig. 8c)  
2 and TP5(3)-l-Dy (Fig. 8d), respectively. The Schiff-base reactions of TP5(1)-l-Dy and  
3 TP5(3)-l-Dy are similar. The rate-limiting step of both reactions is the dehydrogenation  
4 from the amino groups of TP5 (the first step), which needs to overcome an energy  
5 barrier of 53.15 and 53.24 kcal/mol, respectively. The reaction process to yield TP5(2)-  
6 l-Dy is more complex than those of the two other reactions. It begins with  
7 dehydrogenation from the amino group of TP5. But the H atom tends to bond with the  
8 adjacent oxygen atom (O2), and the process needs to overcome an energy barrier of  
9 37.2 kcal/mol. In the second step, the hydrogen proton transfers from O2 to O1, which  
10 needs an energy of 10.7 kcal/mol. The formation of -C-NH bond is thermodynamically  
11 favorable. Subsequently, the cleavage of oxhydryle needs to overcome a barrier of 26.4  
12 kcal/mol. Thus, the rate-limiting step of the reaction needs to overcome a barrier of 37.2  
13 kcal/mol, which is only 0.9 kcal/mol higher than that of the Schiff-base reaction  
14 between CMCS and Dy. Thus, the -C=O group adjacent to the -NH<sub>2</sub> of TP5 could  
15 promote the Schiff-base reaction between TP5 and Dy to form TP5(2)-l-Dy. Moreover,  
16 the two other -NH<sub>2</sub> sites of TP5 may also participate in the Schiff-base reaction in spite  
17 of higher energy barrier. Hence, although the energy barrier of TP5-l-Dy is higher than  
18 that of CMCS-l-Dy, both reactions proceed concomitantly in a competitive way.  
19 Furthermore, it is worth noting that the gelation time of blank hydrogel without TP5 is  
20 shorter than that of hydrogel containing TP5 (data not shown). Therefore, the presence  
21 of TP5 affects the crosslinking between CMCS and Dy because TP5 reacts  
22 competitively with Dy and formation of TP5-l-Dy does not contribute to the  
23 crosslinking due to steric effect. When TP5 is mixed with Dy, it can theoretically and  
24 experimentally react with Dy via Schiff base reaction, which is in full agreement with  
25 the results obtained by FT-IR (Fig. 1), NMR (Fig. 2), and SEM (Fig. S4), and TP5  
26 release (Fig. 6 and 7).

27

#### 28 **4. Conclusion**

1 This work focused on the synthesis and characterization of a novel drug delivery system  
2 based on dynamic hydrogels for the sustainable release of an immunostimulant –  
3 thymopentin (TP5). The hydrogels were prepared via *in situ* gelation of O-  
4 carboxymethyl chitosan with a dynamer made from Jefamine and BTA in aqueous  
5 solution by imine bonding, in the presence or absence of TP5. These hydrogels display  
6 a porous structure, outstanding rheological properties and excellent pH-sensitive  
7 swelling performance. Analyses showed that TP5 is partly attached to the hydrogel  
8 network by imine bonding, together with free TP5 molecules adsorbed by physical  
9 forces into the hydrogel pore walls. *In vitro* release showed a burst release in the first  
10 24 h, followed by a slower release to reach a plateau up to 168 h. The maximum release  
11 rate depends on various factors such as drug content, drug loading and release  
12 conditions. Data show that only free TP5 is released under *in vitro* conditions.  
13 Covalently attached TP5 is not released due to the stability of imine bonds at pH 7.4.  
14 Density functional theory study allowed to better understand the chemical combination  
15 between the hydrogel's components and the drug. All these findings suggest that *in situ*  
16 gelation of water soluble CMCS with a 3D dynamer is a means of choice to design  
17 efficient release systems of hydrophilic drugs, in particular those containing amino  
18 groups which can be covalently attached to the hydrogel network. Last but not least,  
19 this study offers a simple and environmentally friendly strategy to prepare bioactive  
20 and biodegradable hydrogels for drug delivery systems.

21

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26

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